# Isolation, Characterisation, Modification and Application of Fucoidan from *Fucus vesiculosus*

Von der Fakultät für Lebenswissenschaften

der Technischen Universität Carolo Wilhelmina

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*Das, wobei unsere Berechnungen versagen, nennen wir Zufall.*

*Albert Einstein (1879-1955)*

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# Abstract

Fucoidan is a natural occurring, heterogeneous sulphated marine polysaccharide from brown algae. Due to its special structure it is said to possess interesting biological activities. During the implementation of this project, methods were developed to extract fucoidan from *Fucus vesiculosus* (bladder wrack), analyse it, modify it and test it concerning the different biologic activities. The main focus point was to find hydrolytic enzymes as the special sulphatation pattern of the fucoidan may be destroyed by unspecific (e.g. chemical) hydrolysis.

In the beginning, the algae were collected at the beach of Wilhelmshaven, Germany (N 53°51', E 8°14') and the extraction procedure as well as the purification steps were optimised with regard to the yield of these valuable polysaccharides. As the proportion of fucoidan in the algae is subject to seasonal variations, a yield of 1% could be obtained. Two big fractions of fucoidan were produced. Due to the special heterogeneous structure of the polysaccharides, it was very difficult to characterise them. During the project, several methods for detection were established and developed. The combination of these methods allowed a clearer picture of the produced fucoidans to be drawn. By means of a special carbohydrate gel electrophoresis (C-PAGE), size exclusion chromatography (SE-HPLC), colorimetric tests, elemental analysis as well as a combination of gas chromatography (GC) and mass spectrometry (MS), the high molecular fucoidan fraction (FVEhigh), could be elucidated to be 1,300 kDa. The sulphatation degree of this fraction was estimated to be 8.45%. The other fraction (FVElow) was 30-50 kDa in size and had a sulphatation grade of 2.62%. For comparison, commercial fucoidan (Fucoidan Sigma) was estimated to 300 kDa in size and a sulphatation grade of 7.9%. Determination of the monosaccharide composition revealed, that FVEhigh was composed of 78% of fucose and only minor parts of xylose (3%), mannose (1%) and galactose (7%). FVElow, however, consisted of only 59% fucose, 4% xylose, 9% mannose and 4% galactose.

As fucoidan is said to possess so many interesting bioactivities, several of these were tested during the work for this thesis. Initial tests concerning blood coagulation showed an elongation of the blood coagulation time in the Hepato-Quick test for the high molecular weight fucoidans. This allows the use of our self-extracted fucoidan as anti-thrombolytic agent, e.g. to make up for heparin. Further analyses are needed to verify these measurements. Analysis against human cytomegalovirus (HCMV) showed that FVEhigh

as well as Fucoidan Sigma exhibited a very high anti-viral activity. The $IC_{50}$-value, describing the value at which virus load is decreased to 50%, is only 4 µg/ml for FVEhigh and 13.3 µg/ml for Fucoidan Sigma. A therapeutically used virusstatic (ganciclovir; e.g. Cymeven®, Roche) has an $IC_{50}$-value of 14 µg/ml. FVElow has an $IC_{50}$-value of 64 µg/ml and can thus also be considered as an active compound. Additional tests showed an anti-tumoral effect of the self-extracted fucoidans and of Fucoidan Sigma. At *in vivo* analyses in mice a TPA-induced skin cancer was diminished by 10% by fucoidan feeding. *In vitro* tests with Raji-cells showed an inhibition of the induction of Epstein-Barr-Virus early antigen by TPA. To use fucoidan in therapy, secured detection of the polysaccharide is required. One possibility for detection is a specific antibody. During this work, a Fucoidan Sigma antibody was found. This antibody could potentially be used to detect fucoidan in a patient. Further analysis evoking antibodies against FVEhigh and FVElow are of great interest.

Another part of this work deals with the cultivation of microorganisms possessing a fucoidan-degrading potential. At the beginning of the project the fungus *Dendryphiella arenaria* TM 94 was available and was said to possess such an ability on solid-state-media. Our measurements did not show any effects on this special medium as analysis was hampered due to the high monosaccharide concentration of the medium. Additionally, solid-state-media are inappropriate as media for industrial cultivation processes for the production of enzymes. Cultivation experiments with *Dendryphiella arenaria* TM 94 in liquid media were optimised regarding the production of biomass. Additionally, around 80 fungi were isolated from the algae *Fucus vesiculosus*. Two selected isolates (WHV012 and WHV059) as well as the commercially available bacteria *Saccharophagus degradans*, *Pseudoalteromonas atlantica*, *Pseudoalteromonas carrageenovora*, and *Pedobacter heparinus* were tested for their fucoidan-degrading potential. Unfortunately, the activity of *Dendryphiella arenaria* TM 94 could not be definitely reproduced and only hints of a fucoidan-degrading ability were observed for the other strains. Best results were achieved with *Saccharophagus degradans* and *Pseudoalteromonas atlantica* both metabolising FVEhigh. Additionally, FVElow was degraded by a cell disruption solution of *Pseudoalteromonas atlantica.* These results indicate, that the enzyme is intracellular or membrane-bound. Until the end of this work, no fucoidanase had been isolated.

By now, a higher variety of microorganisms with a fucoidan-degrading potential is (commercially) available. With the established and developed methods of this thesis it is possible to perform experiments on the fucoidan degradation. Genetic analyses may help to find a fucoidanase. This opens up new vistas to modify fucoidan and to develop the postulated bioactive potentials. Another important step is the development of an antibody against the FVEhigh and FVElow fractions to analyse the medical advantages.

# Zusammenfassung

Fucoidan ist ein natürlich vorkommendes heterologes sulfatiertes marines Polysaccharid aus Braunalgen, dem aufgrund seiner speziellen Struktur interessante biologische Aktivitäten nachgesagt werden. Im Rahmen dieser Arbeit wurden Möglichkeiten erarbeitet, Fucoidan aus der Braunalge *Fucus vesiculosus* zu gewinnen, zu analysieren, zu modifizieren und auf unterschiedliche biologische Aktivitäten hin zu untersuchen. Bei der Modifikation wurde das Hauptaugenmerk auf das Auffinden hydrolytischer Enzyme gelegt, da die spezielle Sulfatierung der Fucoidane durch unspezifische (z.B. chemische) Hydrolyse zerstört werden kann.

Zunächst wurden dazu die Algen am Strand von Wilhelmshaven (N 53°51', E 8°14') gesammelt und die Vorgehensweise der Extraktion und Aufreinigung im Hinblick auf die Ausbeute dieser wertvollen Polysaccharide optimiert. Es konnten bei einer Gesamtausbeute von 1% (der Anteil an Fucoidan in der Alge ist saisonal unterschiedlich) zwei große Fraktionen von Fucoidanen gewonnen werden. Aufgrund der speziellen heterologen Struktur ist es schwierig, die Polysaccharide zu charakterisieren. Im Laufe des Projekts wurden verschiedene Detektionsmethoden etabliert und weiterentwickelt, damit in Korrelation miteinander ein klares Bild der Fucoidane entstehen konnte.

Mittels einer speziellen Polysaccharid-Gelelektrophorese (C-PAGE), Größenausschluss-chromatographie (SE-HPLC), kolorimetrischen Tests, Elementaranalyse sowie einer Kombination aus Gaschromatographie (GC) und Massenspektrometrie (MS) konnten zwei Fucoidane identifiziert werden. Mit FVEhigh eine ca. 1.300 kDa große Fraktion mit einem Sulfatierungsgrad von 8,45% und mit FVElow eine Fraktion von 30-50 kDa, die einen deutlich geringeren Sulfatierungsgrad von 2,62% aufwies. Als Vergleich wurde ein kommerziell erhältliches Fucoidan aus *Fucus vesiculosus* (Fucoidan Sigma) analysiert. Dieses Fucoidan hatte eine Größe von ca. 300 kDa und einen Sulfatierungsgrad von 7,9%. Eine Analyse der Monosaccharide zeigte, dass FVEhigh zu 78% aus Fucose besteht, FVElow allerdings nur zu 25%. Bei FVElow war der Glucosegehalt mit 59% deutlich erhöht. Weitere detektierbare Monosaccharide waren in beiden Fraktionen Galactose (7 und 4%), Xylose (3 und 4%) sowie Mannose (1 und 9%).

Aufgrund der in der Literatur beschriebenen interessanten Bioaktivitäten wurden unterschiedliche Tests durchgeführt. Tests mit dem selbst isolierten Material zur

Blutgerinnung zeigten eine Verlängerung der Gerinnungszeit im Hepato-Quick-Test bei den höhermolekularen Fucoidanen. Dies ermöglicht den Einsatz von Fucoidan als Anti-Thrombolytikum, z.B. als Ersatz von Heparin. Um gesicherte Aussagen treffen zu können, sollten zusätzliche Analysen durchgeführt werden. Bei der Analyse der Aktivität gegen Humanen Cytomegalovirus (HCMV) zeigte sowohl FVEhigh als auch Fucoidan Sigma eine sehr gute antivirale Aktivität. Der $IC_{50}$-Wert, der Wert, bei dem die Viruslast nur noch 50% beträgt, war für FVEhigh bereits bei 4 µg/ml erreicht. Ein bereits therapeutisch eingesetztes Virusstatikum (Ganciclovir; z.B. in Cymeven®, Roche) hat einen $IC_{50}$-Wert von 14µg/ml. Auch Fucoidan Sigma zeigte einen guten Wert von 13,3 µg/ml und mit FVElow konnte die Viruslast bei 64 µl/ml auf die Hälfte reduziert werden. Weitere Tests zeigten eine anti-tumorale Wirkung der selbst-extrahierten Fucoidane und des Fucoidan Sigma. Bei *in vivo* Analysen an Mäusen konnte ein durch TPA-induzierter Hautkrebs durch die Fütterung von Fucoidan um ca. 10% veringert werden. *In vitro* Tests an Raji-Zellen zeigten eine Inhibierung der Induzierierung des Epstein-Barr-Virus frühen Antigens durch TPA.

Um Fucoidan allerdings wirksam als Therapeutikum einsetzen zu können, ist eine gesicherte Detektion des Polysaccharids erforderlich. Eine Möglichkeit zur Detektion wäre ein spezieller Antikörper. Während dieser Arbeit konnte ein Antikörper gegen Fucoidan Sigma gefunden werden. Mit diesem wäre es -vorausgesetzt eine ausreichende Produktion wäre gewährleistet- möglich, Fucoidan (in Patienten) zu detektieren. Weitere Analysen, die auch Antikörper gegen FVEhigh, FVElow hervorbringen könnten, wären von großem Interesse.

Ein weiterer Teil der Dissertation beschäftigt sich mit der Kultivierung von Mikroorganismen, die ein Fucoidan-abbauendes Potential besitzen. Zu Beginn der Arbeit lag bereits der Pilz *Dendryphiella arenaria* TM 94 vor, dem ein solches Potential auf solid-state-Medium nachgesagt wurde. Eigene Messungen auf diesem Medium zeigten allerdings keine Effekte, da die Analyse durch den hohen Gehalt an Monosacchariden im Medium gestört wurde. Zudem eignet sich ein solid-state Medium nicht für einen industriellen Großprozess zur Produktion von Enzymen aus Mikroorganismen. Kultivierungen mit *Dendryphiella arenaria* TM 94 auf Flüssigmedium konnten im Bioreaktor auf eine hohe Biomasse hin optimiert werden.

Weiterhin wurden ca. 80 Pilze aus der Alge *Fucus vesiculosus* isoliert und zwei ausgewählte Isolate (WHV012 und WHV059) sowie die kommerziell erhältlichen

Bakterienstämme *Saccharophagus degradans*, *Pseudoalteromonas atlantica, Pseudoalteromonas carrageenovora* und *Pedobacter heparinus* auf ihre Fucoidan-abbauende Aktivität getestet. Leider ließ sich die Fuoidan-abbauende Aktivität von *Dendryphiella arenaria* TM 94 nicht eindeutig reproduzieren und auch die anderen Stämme zeigten lediglich Hinweise darauf, dass sie in der Lage sind, Fucoidan abzubauen. Die besten Ergebnisse wurden bei der Verstoffwechselung von FVEhigh von *Saccharophagus degradans* und *Pseudoalteromonas atlantica* sowie mit einer Zellaufschlusssuspension von *Pseudoalteromonas atlantica* auf FVElow erzielt. Dies weist auf ein intrazelluläres oder membrangebundenes Enzym hin. Bis zum Ende der Arbeit konnte keine Fucoidanase isoliert werden.

Zum jetzigen Zeitpunkt sind eine größere Vielfalt an Mikroorganismen (kommerziell) verfügbar, die ein Fucoidan-abbauendes Potential besitzen. Mit den in dieser Arbeit etablierten Methoden können nun Experimente zum Fucoidan-Abbau durchgeführt werden. Genetische Analysen dieser Mikroorganismen können helfen, eine Fucoidanase zu finden. Dadurch würde sich eine Möglichkeit eröffnen, Fucoidan gezielt zu modifizieren und die gezeigten bioaktiven Potentiale noch zu verbessern. Ein wichtiger Schritt ist dabei die Entwicklung von Antikörpern für die FVEhigh und FVElow Fraktionen, um den medizinischen Nutzen weiter untersuchen zu können.

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# 1 State of the Art

## 1.1 Literature Overview

As the first isolation of 'fucoidin' was described in 1918 (Kylin, 1918), this research field is a relatively new one. The number of publications concerning fucoidan started to increase in the beginning of the 1970s and this trend still continues. In the last ten years the research field for fucoidan-degrading enzymes, so called fucoidanases, has come into focus and will be investigated further. This development can be seen in Figure 1 (Holtkamp et al., 2009).

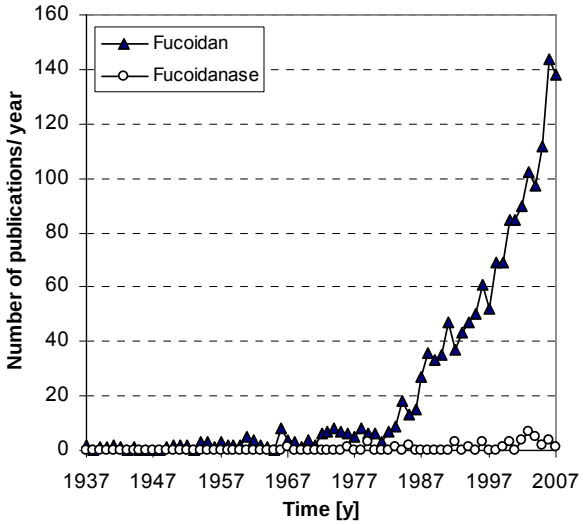



**Figure 1: Number of publications about fucoidan and fucoidanases (data collected by SciFinder® Scholar, American Chemical Society 2007)**

Unfortunately only about 70% of this information is available in English, with the rest published in either Chinese, Japanese or Russian. This makes it very difficult to obtain all possible information in this quite small research field. Regarding the fucoidanases the partition is even more evident. Only half of the available publications is written in English (data collected by SciFinder® Scholar, American Chemical Society 2006).

During the implementation of this project from February 2005 until February 2008 around 300 new publications were published around fucoidan and other algal polysaccharides, showing the increasing importance of this study field.

### 1.1.1 Terminology (Berteau and Mulloy, 2003)

The nomenclature of fucoidan has evolved through several steps. In 1918 Kylin (Kylin, 1918) baptised his sulphated polysaccharide 'fucoidin'. This name was changed by

McNeely (Berteau and Mulloy, 2003; McNeely, 1959) in 1959 to 'fucoidan' to follow the usual polysaccharide nomenclature. As the extracted polysaccharides differed in their composition due to seasonal variations, local climate conditions (Black, 1954) and algal species (Percival and Ross, 1950), it was very difficult to determine if the other mono sugars such as xylose, mannose, galactose and uronic acids were part of the fucoidan or if they were just contaminants. The term 'fucoidan' was even suppressed by some authors due to this uncertainty (Larsen et al., 1966). To circumvent these problems, the new term 'fucans' was used to describe all polysaccharides rich in L-fucose (Percival and Ross, 1950). New techniques for separation and analysis made it possible to distinguish between the different types of sulphated polysaccharides, limiting the term 'fucoidan' to sulphated polysaccharides containing a homofucose backbone. However, not all authors stick to this terminology and some still use the outdated 'fucoidin' or even worse, create their own nomenclature such as 'fucansulfate'(Duarte et al., 2001; Trento et al., 2001).

Berteau and Mulloy (Berteau and Mulloy, 2003) recommended the use of 'sulfated fucan' to describe a polysaccharide mainly based on sulphated fucose, with less than 10% other monosaccharides. This term was applied to the sulphated fucans of marine invertebrates (Alves et al., 1998; Ribeiro et al., 1994; Vilela-Silva et al., 1999), whereas the term 'fucoidan' has been reserved for fucans isolated from algae. To keep confusion to a minimum, this terminology is adopted to this thesis.

### 1.1.2 Sources and Characterisation of Fucoidan

*Sources*

Sulphated polysaccharides can be found in various marine sources. This might be sea cucumber (Ribeiro et al., 1994), sea urchin (Mulloy et al., 1994; Vilela-Silva et al., 1999) or brown algae (Descamps et al., 2006). In recent years many different algae and invertebrates have been analysed for their content of fucoidans including *Fucus vesiculosus* (Beress et al., 1993; Obluchinskaya and Minina, 2004; Wu et al., 2002), *Sargassum stenophyllum* (Duarte et al., 2001), *Chorda filum* (Bakunina et al., 2002), *Ascophyllum nodosum* (Medcalf and Larsen, 1977), *Cladosiphon okamuranus* (Sakai et al., 2003b), *Dictyota menstrualis* (Albuquerque et al., 2004), *Fucus evanescens* (Bakunina et al., 2002; Bilan et al., 2006; Kuznetsova et al., 2003), *Fucus serratus* (Bilan et al., 2006), *Fucus distichus* (Bilan et al., 2004), *Caulerpa racemosa* (Ghosh et al., 2004), *Hizikia fusiforme* (Li et al., 2006), *Padina gymnospora* (Usov et al., 2004), *Kjellmaniella*

*crassifolia* (Sakai et al., 2002) and *Analipus japonicus* (Bilan et al., 2007). Some of these sources are shown in Figure 2.

All these different sources contain different forms of fucoidan. They have to be extracted in special ways in order to obtain high yields. Many of the research groups extract fucoidan on their own and subsequently analyse the properties of their extracts (Obluchinskaya and Minina, 2004). Up to now there is only one commercially available fucoidan. This is fucoidan from *Fucus vesiculosus* (bladder wrack; Figure 2-1).


**Figure 2: Sources of fucoidan: 1: *Fucus vesiculosus*, 2: *Laminaria digitata*, 3: *Fucus evanescens*, 4: *Fucus serratus*, 5: *Ascophyllum nodosum*, 6: *Pelvetia canaliculata*, 7: *Cladosiphon okamuranus*, 8: *Hizikia fusiforme*, 9: *Laminaria japonica*, 10: *Sargassum horneri*, 11: *Nemacystus decipiens*, 12: *Padina gymnospora*, 13: *Stichopus japonicus***

*Function for the Algae*

Although the function of fucoidan for the algae itself has not been thoroughly investigated, there are several theories on the subject. The fucoidan content differs between the intertidal zone (high amounts of fucoidan) and the zone under the low water line (less amounts of fucoidan). A conservation against dehydration can thus be assumed (Black et al., 1952). Another suggestion is the enhancement of cell wall stability (Mabeau et al., 1990). This could also be supported by the discovery that the sugar content of the algae gradually increases from April to September (Honya et al., 1999), during which time the algae are exposed to higher amounts of sunlight whose UV light may destroy cell constituents.

*Characterisation*

As fucoidan is a heteropolysaccharide and its composition differs from source and season (Black, 1954), it is inevitable to determine the molecular components before usage. The first suggestion for a fucoidan structure was made by Percival and Ross for fucoidan from *Fucus vesiculosu*s in 1950 (Percival and Ross, 1950). Not before 1993 Patankar (Patankar et al., 1993) successfully elucidated this structure and described it as a polysaccharide consisting mainly of α-1,3-L-fucose. The main differences in fucoidans originate from their source. Fucoidans from invertebrates show a linear backbone of sulphated monosaccharides whereas algal fucoidans may be branched in various ways. The elucidation of the structure is not concluded yet and thus the algae cannot be grouped by their fucoidan structure. However some similarities can be described:

Most of the algae contain polysaccharides that consist mainly of sulphated L-fucose with a fucose content of 34-44% (Kloareg et al., 1986). Other common sugars include galactose, mannose, xylose and uronic acids (Duarte et al., 2001; Mian and Percival, 1973a; Mian and Percival, 1973b). The sulphation may occur at position 2, 3 and 4 and the monosaccharides are associated via α-1,2, α-1,3 or α-1,4 glycosidic bonds. The sulphatation degree differs with the location and season of collection and ranges between 4-8% (Black, 1954).

The molecular weights of fucoidans depends also on the source from which they are obtained. There is a high variety between the smaller molecular weights like 13kDa and 950 kDa (Li et al., 2006) as shown in Table 1.

**Table 1: Molecular Weight Distribution Among Fucoidans**

| Molecular Weight of Fucoidan | Source | Reference |
|---|---|---|
| 13 kDa | *Ascophyllum nodosum* | (Daniel et al., 2001) |
| 16 kDa | *Ascophyllum nodosum* | (Senni et al., 2006) |
| 25 kDa | *Hizikia fusiforme* | (Li et al., 2006) |
| 100-180 kDa | *Fucus vesiculosus* (Sigma) | (Suppiramaniam et al., 2006) |
| 160 kDa | *Fucus vesiculosus* | (Ruperez et al., 2002) |
| 189 kDa | *Laminaria japonica* | (Zhang et al., 2005) |
| 200 kDa | *Cladosiphon okamuranus* | (Sakai et al., 2003a) |
| 950 kDa | *Hizikia fusiforme* | (Li et al., 2006) |

### 1.1.3 Bioactivity of Fucoidan

Fucoidans and their oligosaccharides are attributed several different bioactivities. These include anti-tumoral (Siddhanta and Murthy, 2001), anti-coagulant (Dobashi et al., 1989;

Farias et al., 2000; Grauffel et al., 1989; Silva et al., 2005), anti-viral (Baba et al., 1988; Lapshina et al., 2006; Lee et al., 2004b; Witvrouw and De Clercq, 1997) and anti-inflammatory (Siddhanta and Murthy, 2001) activities. These many potential applications make the fucoidans such an interesting research object.

It is postulated that sulphate groups are essential for the antiviral activity and that a higher sulphation degree is beneficial for the antiviral (Qiu et al., 2006; Witvrouw and De Clercq, 1997) and anti-tumoral (Koyanagi et al., 2003) activity and that structure plays a major role in the biological activity (Boisson-Vidal et al., 2000). Algal polysaccharides have been suggested to affect the virus adsorption and penetration (Damonte et al., 2004). The anti-tumour activity relies on the inhibition of the proliferation and the induction of apoptosis (Aisa et al., 2005). Wound healing processes are accelerated because of the activity of fucoidan on the collagen gel contraction (Fujimura et al., 2000). Even antibodies against fucoidans have been described (Nakagawa et al., 2000). They can be used as a detection tool for fucoidan in a patient.

### 1.1.4 Applications of Fucoidan and its Oligosaccharides

As fucoidan has so many interesting properties there is a wide range of applications. Some of these applications of native fucoidans are shown in Table 2. Applications of processed fucoidan are shown in Table 3.

**Table 2: Applications of native fucoidan (Holtkamp et al., 2009)**

| Fucoidan source | Application | Reference |
|---|---|---|
| *Ascophyllum nodosum* | Modulation of connective tissue proteolysis | (Senni et al., 2006) |
| *Fucus evanescens* | Formation of virus in the cells of tobacco leaves | (Lapshina et al., 2007) |
| *Fucus vesiculosus* (from Sigma) | Inhibition of cellular and neurotoxic effects in rat | (Jhamandas Jack et al., 2005) |
| *Fucus vesiculosus* (from Sigma) | AMPA receptors | (Suppiramaniam et al., 2006) |
| *Fucus vesiculosus* (from Sigma) | Cell apoptosis | (Aisa et al., 2005) |
| *Fucus vesiculosus* (from Sigma) | Delay of thrombus growth | (Thorlacius et al., 2000) |
| *Fucus vesiculosus* (whole algae) | Menstrual cycle length | (Skibola Christine, 2004) |
| *Laminaria japonica* | Inhibition of the development of proteinuria | (Zhang et al., 2005) |
| *Laminaria japonica, Fucus evanescens, Laminaria cichorioides* | Development of sea urchin embryos | (Kiseleva et al., 2005) |
| *Undaria pinnatifida* | Antiviral effects | (Lee et al., 2004a) |

**Table 3: Applications of processed fucoidan (Holtkamp et al., 2009)**

| Fucoidan source | Application | Reference |
|---|---|---|
| *Ascophyllum nodosum* (radical depolymerisation process) | Prevention of neointimal hyperplasia | (Deux et al., 2002) |
| *Pelvetia canaliculata* (enzymatically cleaved by an endo-fucanase preparation from a marine bacterium (*Flavobacteriaceae*) | Systemic resistance against tobacco mosaic virus | (Klarzynski et al., 2003) |

Another interesting idea would be to apply fucoidan as a drug release system (Sezer and Akbuga, 2006).

## 1.1.5 Modification of Fucoidans

### 1.1.5.1 Fucoidanases – Fucoidan-Degrading Enzymes

EC number 3.2.1.44 describes the poly (1,2-alpha-L-fucose-4-sulfate) glycanohydrolase also known as α-L-fucosidase. The enzyme belongs to the glycoside-hydrolysing hydrolases (3.2) like amylases. The name fucoidanase, used for enzymes cleaving fucoidans, is taken as a synonym for α-L-fucosidase so far. The enzymes can be both intra- and extracellular. However, no commercial endofucosidase is available yet (Chevolot et al., 1999). To find fucoidan-degrading enzymes one can search in almost every marine polysaccharide-containing plant such as algae (Bakunina et al., 2000; Barbeyron et al., 2001) and also in sources with other high molecular polysaccharides such as pulp production sites (Descamps et al., 2006). Other successfully exploited sources are salt marsh grass (Andrykovitch and Marx, 1988; Ekborg et al., 2005), sand (Wu et al., 2002), sea cucumber (Bakunina et al., 2000) or sponges and molluscs (Daniel et al., 2001).
One can distinguish between two kinds of fucoidanases. The endofucoidanase cleaves inside the molecule whereas the exofucoidanase cleaves off oligosaccharides from the ends of the polysaccharide chain, leading to lower molecular weight fucoidans. The high variety of fucoidan sources requires a large number of cleaving patterns. Therefore there is no possibility to describe 'the unique fucoidanase', but only a group of hydrolysing enzymes. The exact mechanism of these cleavages remains unknown to this day.

*Genetic Characterisation of Fucoidanases*

Very little is known about the genes encoding fucoidanases. In 2006 Colin (Colin et al., 2006) described the cloning and biochemical characterization of a special fucanase. It was shown that the enzyme is a sulphated fucan α-1,4-endohydrolase with five domains, thus

constituting a new family of glycoside hydrolases. Production of fucoidanase genes in potent host organisms could lead to better understanding of the mechanisms of these enzymes.

*Sources for Fucoidan Degrading Bacteria*

In Table 4 several examples for fucoidan-degrading bacteria are presented.

**Table 4: Examples of fucoidan-degrading bacteria in literature; n.s. = not specified**

| Family | Genus | Species | Strain | Isolated from | Enzyme | | | Reference |
|---|---|---|---|---|---|---|---|---|
| | | | | | Type | $T_{opt}$ | pH | |
| *Vibrionaceae* | *Vibrio* | n.s. | N-5 | Sea sand | Fucoidanase | 40 °C | 6.0 | (Furukawa et al., 1992) |
| | | | | | Fucoidan Sulphatase | | 7.5 | |
| *Alteromonadaceae* | n.s. | n.s. | n.s. | Coastal sea water | Endosulphated fucan-digesting Catalyse endo-type | 30-35 °C | 6.5-8.0 | (Sakai et al., 2004) |
| | *Pseudoalteromonas* | *P. citrea* | KMM 3296 | *F. evanescens* | Hydrolysis of O-glycosidic bonds | <50 °C | n.s. | (Bakunina et al., 2002) |
| | | | KMM 3297 | *Apostichopus japonicus* | n.s. | <50 °C | n.s. | |
| | | | KMM 3298 | *Corda filum* | n.s. | <40 °C | n.s. | |
| | *Saccharophagus* | *S.degradans* | 2-40 | *Spartina alterniflora* | n.s. | n.s. | n.s. | (Gonzalez and Weiner, 2000) |
| *Flavobacteriaceae* | *Marini flexile fucanivorans* | n.s. | SW 5 | Water treatment plant | n.s. | 20-25 °C | 7.5 | (Descamps et al., 2006) |
| | *Gramella* | n.s. | KMM 6054 | *Strongylocentrotus intermedius* | n.s. | n.s. | n.s. | (Urvantseva et al., 2006) |
| | *Maribacter* | *M. algae* | n.s. | *Acrosiphonia sonderi* | n.s | n.s. | n.s. | |
| | | | KMM 621 | *S. intermedius* | n.s. | n.s. | n.s. | |
| *Melanconiaceae* | *Hyphomycete* | *Dendryphiella arenaria* | TM 94 | Sea sand | Exo- and endotype hydrolysis | 50 °C | 6.0 | (Wu et al., 2002) |
| *Pectinidae* | *Pectinella* | *Patinopecten yessoensis* | n.s. | Homogenate of hepatopancreas | Fucoidanase | n.s. | IEP 7.4 | (Kitamura et al., 1992) |
| | | | | | α-L-Fucosidase | n.s. | n.s. | |
| | | | | | Arylsulphatase | n.s. | n.s. | |
| | | *Pecten maximus* | n.s. | Digestive glands | Fucosidase EC 3.2.1.51 | 60 °C | | (Berteau et al., 2002) |
| *Littorinideae* | *Littorina* | *L. kurila* | n.s. | Homogenate of hepatopancreas | Fucoidanase | <45 °C | 5.4 | (Kusaykin et al., 2003; Kusaykin et al., 2006) |
| | | | | | α-L-Fucosidase | n.s. | 8.5 | |
| | | | | | Arylsulphatase | n.s. | n.s. | |
| *Sphingomonaceae* | *Sphingomonas* | *Sphingomonas sp.* | PF-1 | Sea water | Fucoidanase (endotype) | n.s. | n.s. | (Kim et al., 2008) |

### 1.1.5.2 Chemical Hydrolysis

So far chemical hydrolysis seems to be the only possibility to produce fucoidan oligosaccharides as no fucoidanases are available yet (Chevolot et al., 1999; Kim et al., 2008). There are several examples in literature on how the chemical degradation and modification of polysaccharides is performed. Already in 1948 Swanson and Cori (Swanson and Cori, 1948) studied the kinetics of the acid hydrolysis of starch-like polysaccharides. Karlsson and Singh (Karlsson and Singh, 1999) described the acid hydrolysis of different sulphated polysaccharides (κ-, ι-, λ-carrageenan, dextran sulphate, heparin) and analysed their sensitivity to desulphatation due to the hydrolysis conditions.
A commonly used method producing bioactive oligosaccharides through chemical hydrolysis has been presented by Yuan and Song (Yuan and Song, 2005). They produced oligosaccharides from carrageenan from *Kappaphycus striatum* to compare the anti-tumour activity with carrageenan polysaccharides. Pomin et al. analysed the selective cleavage and anti-coagulant activity of a sulfated fucan. They regio specifically removed a 2-sulphate ester from the polysaccharide by mild acid hydrolysis and prepared oligosaccharides with an heparin cofactor II-dependent anti-coagulant activity (Pomin et al., 2005).

To evaluate the enzymatic degradation of marine polysaccharides successfully, chemical hydrolysis can thus be a potent tool. With defined conditions, sulphated oligosaccharides can be produced which can be used as standards for analysing the enzymatically degraded polysaccharides.

## 1.2 Aim of this Thesis

As fucoidans and their bioactivities are interesting for research and medicine, the aim of this work is to produce and characterise fucoidan and to find and produce fucoidan-hydrolysing enzymes (Figure 3; green label). These enzymes can be found in marine organisms and should be able to degrade the fucoidans without cleaving the sulphate groups, thus possibly leading to a higher bioactivity. The enzymes shall be characterised with regard to their cleaving pattern and optimal operating conditions. This information is then be used to produce large amounts of bioactive fucoidan oligosaccharides. The challenge in these experiments is that one first has to know the original material (fucoidan that has been extracted from brown algae) very well before one can find the enzymes hydrolysing these polysaccharides in order to evoke the desired bioactivity (Figure 3; green label). This divides the thesis into three major parts:

First, the development and optimisation of an extraction procedure and elucidation of the structure and properties of the fucoidan, received from the brown algae. Second, the application of fucoidan and fucoidan oligosaccharides for specific bioactive problems and third the screening of marine organisms to find potent enzyme producers and the processing of these organisms in order to produce the pure enzyme.

The first two steps (Figure 3; blue labels) are also the two big bottlenecks of this project. The extraction procedure has to be optimised because one has to work with pure material. As algae are a natural product, their compositions have seasonal variations (Black, 1954; Honya et al., 1999) and a quite low content of fucoidan (around 4-8%). The extraction procedure has to take these challenges into account. The commercially available fucoidan does not fulfil all premises and is also very expensive. Additionally, the origin of the algae is not known exactly. *Fucus vesiculosus* can be found at the German coast in large amounts and can easily be exploited. Without a defined raw material it is not possible to start the screening for microorganisms in a proper way. As the fucoidan material is new and unknown in the beginning of the project, a screening method for the microorganisms has to be evaluated. For the bioactivity, several applications have already been named in literature. In this thesis four potential application shall be analysed. As the anti-coagulant properties of fucoidan have been described sufficiently, this is the starting point of bioactivity analyses. Then, as fucoidan is said to possess an anti-viral potential it will be tested on HCMV, which has not been performed before with fucoidan from *Fucus vesiculosus*. To elucidate a new anti-tumour activity, cancer tests shall be performed. The

classification of tumours that can be hampered by fucoidan application is the aim of these analyses as they enhance the available data in this field. As our institute accommodates a very strong antibody research group, the fourth potential for fucoidan application is obvious: It shall be elucidated whether a potent anti-fucoidan-antibody can be found. Figure 3 shows a flow chart of the project setup with the different aims to accomplish as well as the possible pathways to achieve them.

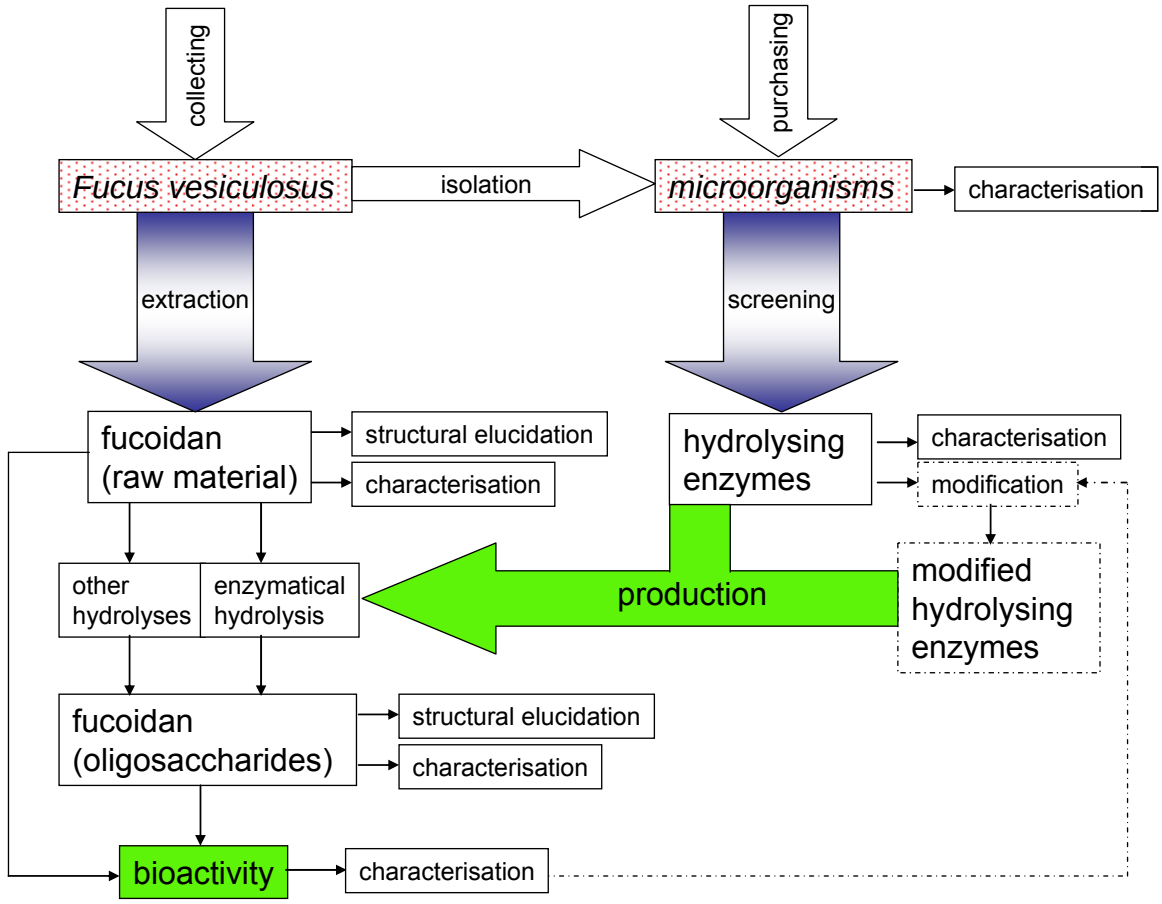



**Figure 3: Project set up**

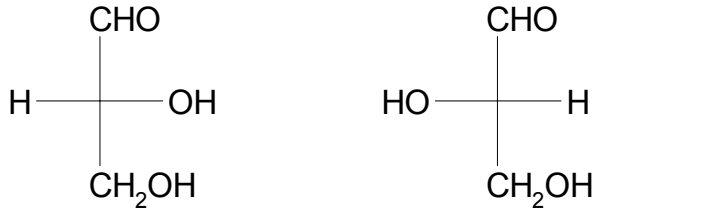
*To summarise the aims of this project:*

A standardised new fucoidan shall be produced from *Fucus vesiculosus* collected at different seasons at the German coast. These heterogeneous polysaccharides are to be characterised for their compounds, structure and size and then used for several new bioactivity applications (e.g. non-tested viruses and new tumour-classes). To produce low molecular weight oligofucoidans, microorganisms shall be found that possess a fucoidan-degrading enzyme (complex) and are able to cleave these special polysaccharides without destroying the unique sulphatation pattern.

# 2 Theoretical Background

## 2.1 Sugar Nomenclature

When discussing polysaccharides the connection between the different monosaccharides and branching thereof is of great importance. Most sugars are chiral and are thus containing at least one stereo centre. To explain the different types, two main representations are common. The Fischer projection is the standard method to describe tetraedrical carbon atoms in a two dimensional way and is thus extremely often used to present carbohydrates. In the Fischer projection, the molecule is drawn as a cross with the carbon atom as intersection of the two axes. Horizontal lines represent linkages that are aiming at the contemplator, vertical lines are pointing away from the contemplator. From this representation it is easy to distinguish between the R and S configuration of the different stereo centres. Although this nomenclature would be sufficient to describe sugars, an older nomenclature is used. The configuration of a sugar is hereby correlated to the two enantiomeres of 2,3-Dihydroxypropanal (glycerolaldehyde) which is the simplest chiral sugar and shown in Figure 4.

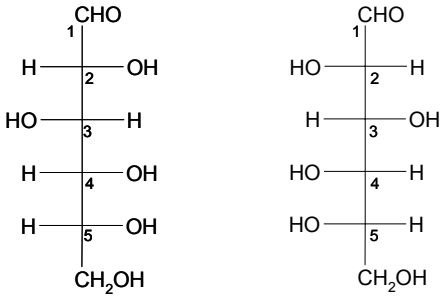



(*R*)-(+)-2,3-Dihydroxypropanal [D-(+)-Glycerolaldehyde]

(*S*)-(+)-2,3-Dihydroxypropanal [L-(+)-Glycerolaldehyde]

**Figure 4: 2,3-Dihydroxypropanal modified after Vollhardt (Vollhardt and Schore, 2005)**

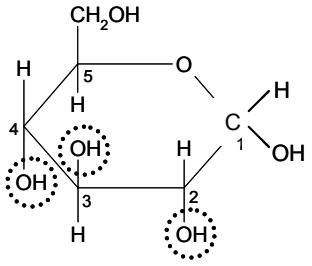
Instead of R and S the prefixes D (for the (+)-enantiomer of glycerolaldehyde) and L (for the (–)-enantiomer are used. This D,L-nomenclature parts the sugars into two serials. To determine whether a sugar is belonging to the D- or the L-serial, the chiral centre which is farthest away from the carbonyl group is of great importance. In the Fischer projection the OH-group is pointing to the right in D-conformation and to the left in L-conformation. The example of glucose is shown in Figure 5. Almost all naturally occurring sugars are D-configured.




D-Glucose L-Glucose

**Figure 5: Fischer projection of glucose modified after Vollhardt (Vollhardt and Schore, 2005)**

Another commonly used representing form is the Haworth projection. This projection is used because sugars form intermolecular half acetals (ring-structures). To display this ring structure the right way, the groups that were displayed to the right in the Fischer projection point to the bottom in the Haworth projection. An example for D-Glucopyranose is shown in Figure 6.




D-Glucopyranose

**Figure 6: Haworth projection modified after Vollhardt (Vollhardt and Schore, 2005)**

After the cyclisation a new chiral centre is formed at the carbonyl-carbon atom. With the half acetal formation two new compounds are built (two diasteromeres that differ in the configuration of the acetal carbon atom). If the acetal carbon atom is S-configurated it is called α. If it is R-configured, it is called β. As this is only found with sugars, these Isomeres are called anomeres, and the new chiral centre is called anomeric carbon atom. Generally it can be said, that the α-bond is directed opposite to the bulky $CH_2OH$ group, the β-bond is on the same side.

To display this configuration both the Fischer projection and the Haworth projection can be applied. In the Fischer projection of the α-form the OH-group of the anomeric carbon atom is directing to the right, in the projection of the β-form the OH group of the anomeric carbon atom is pointing to the left. In the Haworth projection, substituents that are pointing

to the right in the Fischer projection are directed to the bottom. The combination of these two projections is shown in Figure 7.

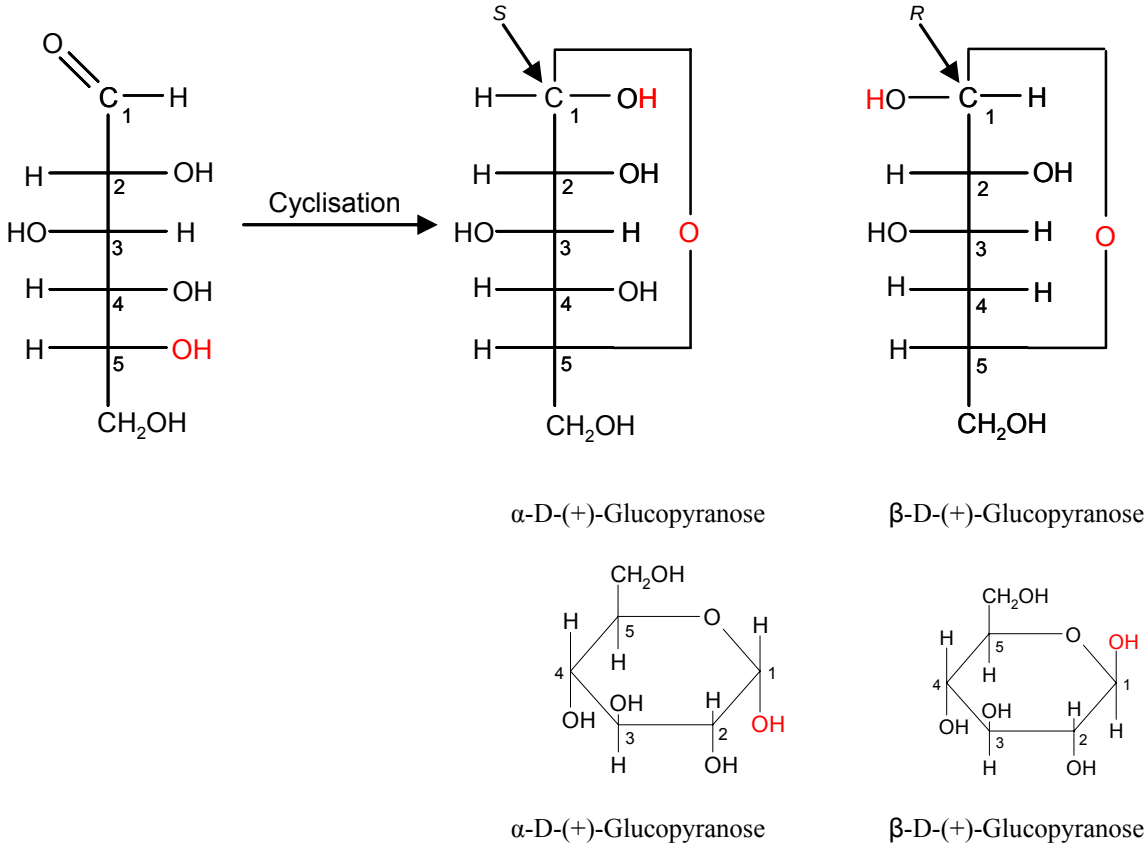



**Figure 7: Fischer-projection of the cyclisation of glycopyranoses modified after Vollhardt (Vollhardt and Schore, 2005)**

According to the presented nomenclature the monosaccharides dealt with in this thesis are presented in Figure 8.

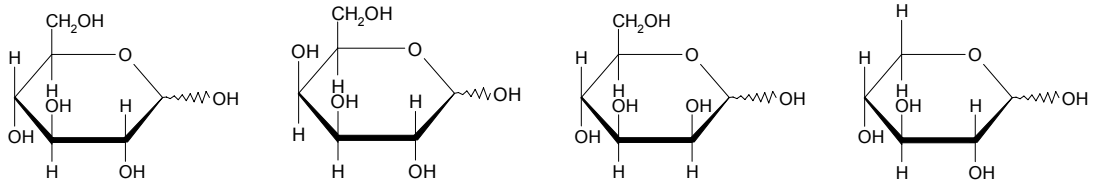



**Figure 8: Important monosaccharides**

Even though it is very common to present sugars in the Haworth projection, the conformation of the sugars is chair-like. The anomeric carbon atom is, in this presentation, by definition displayed in the right edge, the ether oxygen is displayed in the right upper corner. Most aldoses take the chair conformation in which the bulky hydroxymethylgroups (C5) is standing equatorial. Figure 9 shows the different possibilities for glucose.

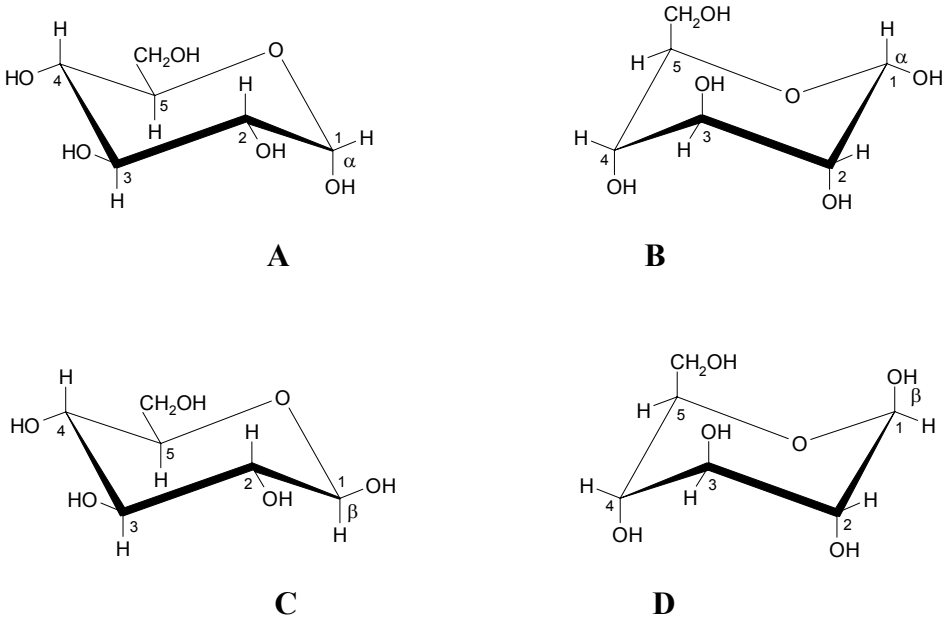



**Figure 9: Different chair conformations of glucose; A and B α-glucose and C and D β-glucose.**

The most important monosaccharide concerning fucoidan is α-L-fucose (6-desoxy-L-galactose). As L-sugars are the mirror image of the according D-sugar, the α- and β-linkages are presented to the opposite side as common with the D-sugars. This is due to the convention that the ether oxygen has to be displayed on the upper right corner and the carbon atom is displayed as the right edge of the molecule. The L-sugar is rotated that long, until these conventions are fulfilled. Figure 10 gives an overview about the most likely presentation forms of α-L-fucose. The chair-conformation (C) is also used in the presentation of the fucoidan polysaccharides (see Figure 15). To keep confusion to a minimum, the linkage- and branching types of the polysaccharides are given in the particular figure.

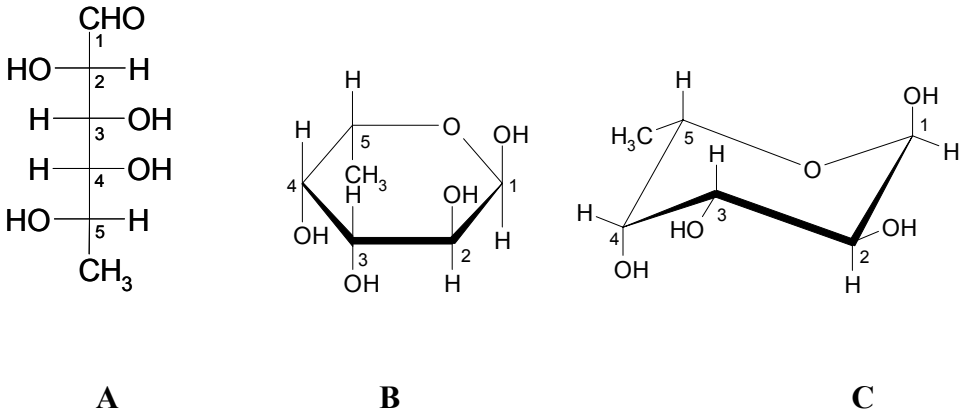



**Figure 10: α-L-fucose presented in Fischer-projection (A), Haworth-projection (B) and chair conformation (C; modified after (Vanhooren and Vandamme, 1999))**

## 2.2 Structures of Interesting (Sulphated) Polysaccharides

In the following paragraphs the structures of interesting (sulphated) polysaccharides are shown. These polysaccharides are important to know, because they share characteristics with fucoidan concerning derivation and/or structural elements.

### 2.2.1 Heparin

Heparin gained great importance in medicine in the application as anti-clotting agent. Its molecular weight ranges between 4 to 40 kDa with a major peak at 15 kDa. The structure of heparins is variable. They consist of alternating D-glucosamino glucans and uronic acids (Lohse and Linhardt, 1992; Shanmugam and Mody, 2000; Shaya et al., 2006; Volpi and Maccari, 2006). The structure can be seen in Figure 11. Linkage is through α-1,4 glycosidic bonds. Natural heparins are gained out of the small intestines of pigs or bovine lung tissue, thus there is a risk of contamination and potent infections like prions. Especially these drawbacks intensify the research on fucoidan and other sulphated polysaccharides that are potent anti-clotting agents (Kim et al., 2003; Shanmugam and Mody, 2000).

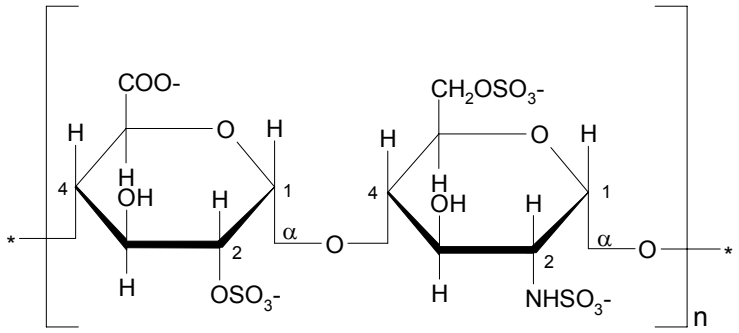



**Figure 11: Structure of heparin modified after Voet (Voet and Voet, 1994)**

Enzymes active on heparin are the so called heparinases I and II (EC number 4.2.2.7). These are heparin-lyases that cleave α-1,4-glycosidic bonds. Heparin sulphate is cleaved by heparinase III, also a heparin lyase (4.2.2.8), leading to the release of sulphate (http:/www.brenda-enzymes.info).

## 2.2.2 Dextran Sulphate

Dextran sulphate is a polymer of sulphated glucose which is connected via α-1,3- and α-1,6-linkages. The variant marketed by Fluka is derived from *Leuconostoc mesenteroides* and is supplied in the sodium salt form to make it soluble and stable in water. Sulphation grade is around 17% which is equivalent to 2.3 sulphate groups per glucosyl residue.

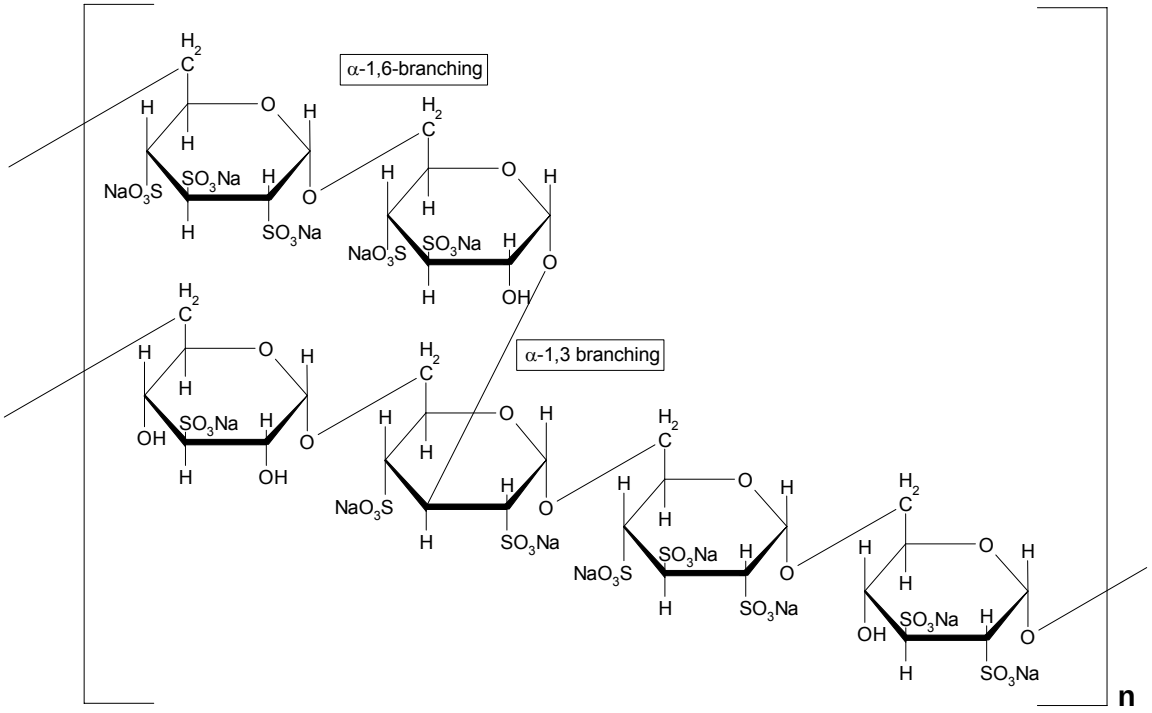



**Figure 12: Structure of Dextran sulphate sodium salt from *Leuconostoc ssp.* (Fluka)**

## 2.2.3 Carrageenan

Carrageenan is a marine polysaccharide extracted from red algae, occurring in several different structure modifications. Six different carrageenan types have been distinguished so far. Classification is based on the sulphation pattern as well as the constituent monomers. The basic structure of carrageenan is linear and consists of repeating dimeric structures of 1,4-β-D-galactopyranosyl-1,3-α-D-galactopyranosyl or in some cases 3,6-anhydro-galactopyranosyl (Falshaw et al., 2005; F-Tischer et al., 2006; Liang et al., 2006). An overview about the different carrageenan structures is shown in Figure 13.

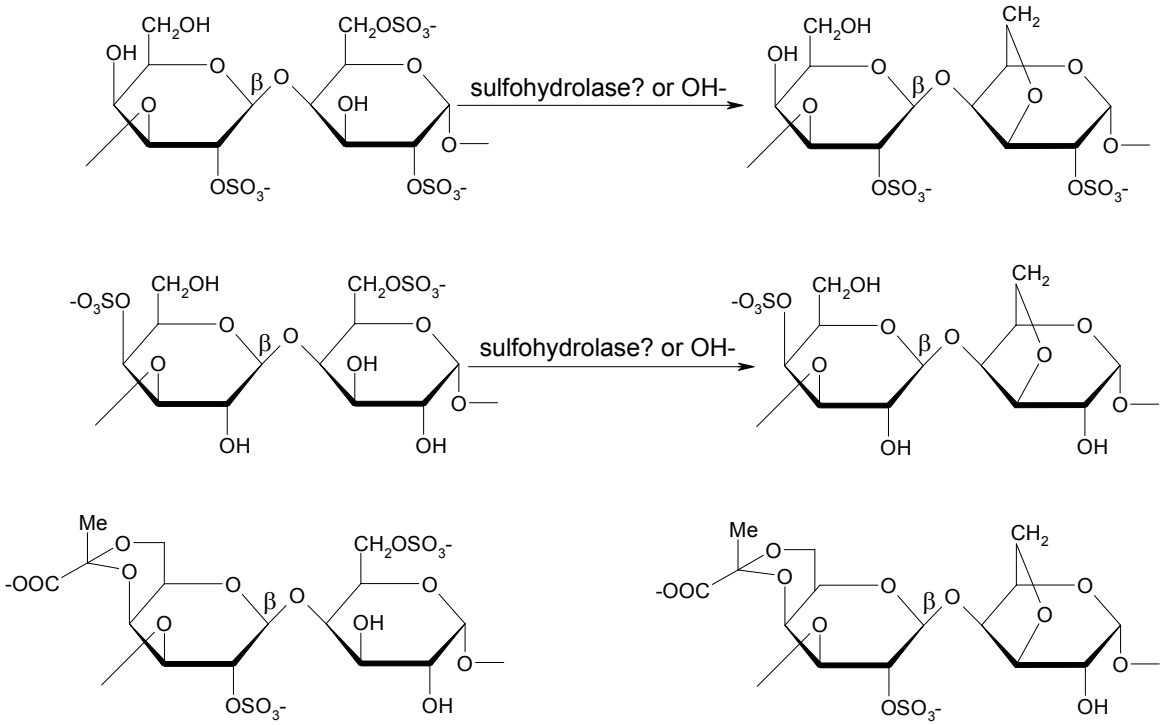



**Figure 13: Structures of Carrageenan Modified after Falshaw (Falshaw et al., 2005)**

An example of an enzyme active on carrageenan is the λ-carrageenase from *Pseudoalteromonas carrageenovora* (active on λ-carrageenan). This enzyme is an endo-carrageenase of about 100 kDa, cleaving β-1,4 linkages to produce mainly tetrasaccharides (Guibet et al., 2007; Ohta and Hatada, 2006). For the other carrageenans similar enzymes have been described. κ-carrageenase (Knutsen and Grasdalen, 1992; Mou et al., 2004), ι-carrageenase (Michel et al., 2003) or a sulfohydrolase catalysing the conversion of μ- into κ-carrageenan (Wong and Craigie, 1978).

### 2.2.4 Laminarin

Besides fucoidan, laminarin is extracted from the brown algae *Laminaria digitata*. It consists of a dimer of two glucose units, being linked through a 1-3 beta glycosidic bond. Laminarin exists as water soluble (from *Laminaria digitata*) and insoluble form (from *Laminaria clouston*) (Harborne and Baxter, 1999). Laminarin consists of D-glucose-units that are linked by β-1,3 and/or β-1,6-bonds (Miao et al., 1995; Nelson and Lewis, 1974; Shanmugam and Mody, 2000). The dimeric structure of laminarin can be seen in Figure 14.

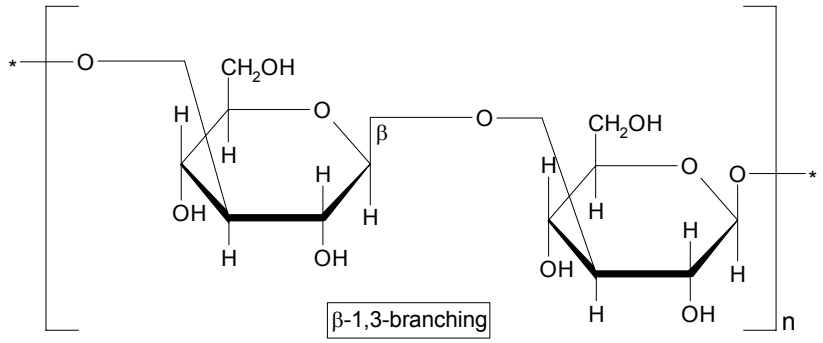



**Figure 14: One structural element of laminarin**

The partial synthetically produced sulphated form of laminarin (laminarin sulphate) has anti-coagulant properties similar to those of fucoidan or heparin (Miao et al., 1995). Enzymes active on laminarin are β-D-glucanases, which are hydrolysing enzymes. Examples of these β-D-glucanases are endo-1,3-β-glucanase (EC number 3.2.1.6), also called laminarinase, glucan-1,3- β-glucosidase (EC number 3.2.1.58), endo-1,3- β-D-glucosidase (EC number 3.2.1.39) and endo-1,6 β-D glucosidase (http:/www.brenda-enzymes.info).

### 2.2.5 Fucoidan

In Figure 15 the typification of two homofucose backbone chains in brown seaweed fucoidan is shown. One chain consists of a regular 1-3 linked homofucose backbone whereas the other chain comprises of alternating 1-3 and 1-4 linked fucose residues. Figure 16 shows three examples of structural motifs of fucoidan. These motifs do not depict the great diversity of fucoidan structures that are possible, but rather show only a small range of common motifs.

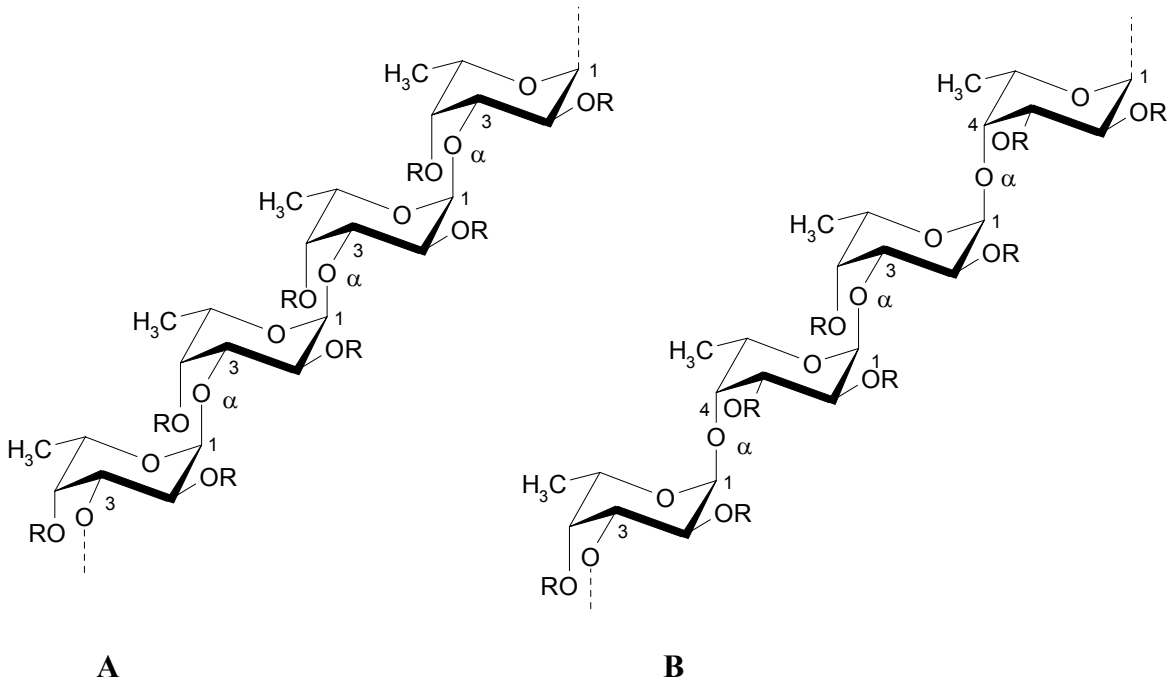



**Figure 15: Typification of two homofucose backbone chains in brown seaweed fucoidan. Modified after Cumashi (Cumashi et al., 2007). A-chains are constructed of only repeating (1→3)-linked α-L-fucopyranose residues whereas B-chains consist of alternating (1-3)- and (1-4)-linked α-L-fucopyranose residues. R shows the places of potential attachment of carbohydrate(α-L-fucopyranose, α-D-glucoronic acid) and non-carbohydrate (sulphate and acetyl groups) substituents (Holtkamp et al., 2009)**

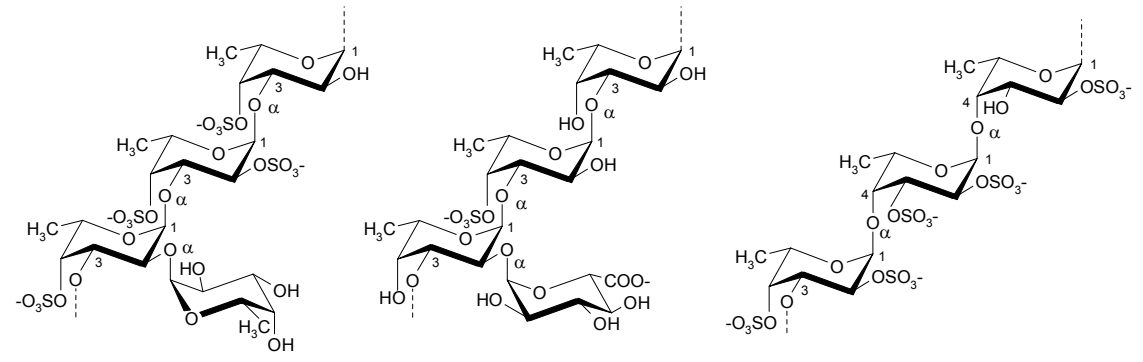



**Figure 16: Structural motifs for fucoidans from three different brown seaweeds. Modified after Cumashi (Cumashi et al., 2007; Holtkamp et al., 2009).**

## 2.3 Methods for Polysaccharide-Analyses

### 2.3.1 SE-HPLC (Size Exclusion High Performance Liquid Chromatography)

The SE-HPLC is a very convenient system for the separation of biological molecules. Size exclusion chromatography uses porous particles to separate these molecules of different sizes. Molecules that are smaller than the pore size can enter the particles and thus have a longer path and longer retention time than larger molecules which are not able to enter the column particles. All molecules larger than the pore size are unretained and eluted together. Molecules that can enter the pores have an average residence time in the particles. This residence time depends on the size and shape of the bio molecules. Hence, the pore size of the column has to be chosen carefully with regard to the expected size of the molecules to be separated. Figure 17 shows the general principle of a SEC. It is important to know, that separation is performed due to the size of the molecules and not the molecular weight. That means, that molecules of the same molecular weight migrate differently in the column due to branching and folding effects. So only relative MW values can be obtained with the same sample type.

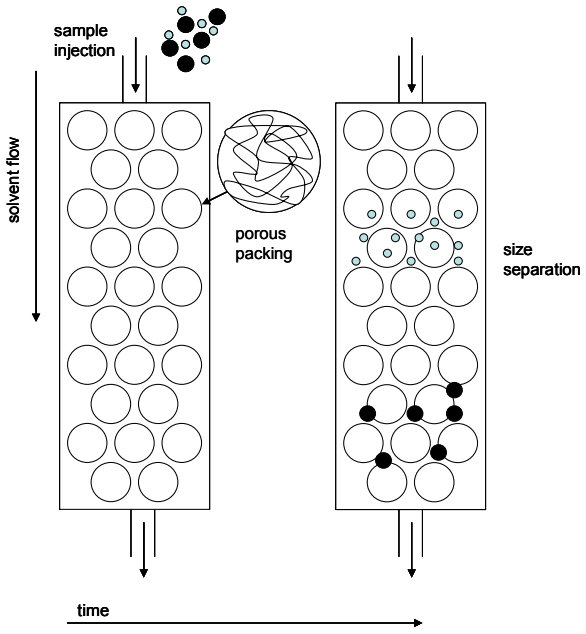



**Figure 17: Size exclusion chromatography principle**

## 2.3.2 Alcian Blue Staining

Alcian Blue is used as a fixative for Glycosaminoglycans in polyacrylamidegels prior to silver staining (Cowman et al., 1984; Min and Cowman, 1986). Figure 18: shows the structure of Alcian Blue.

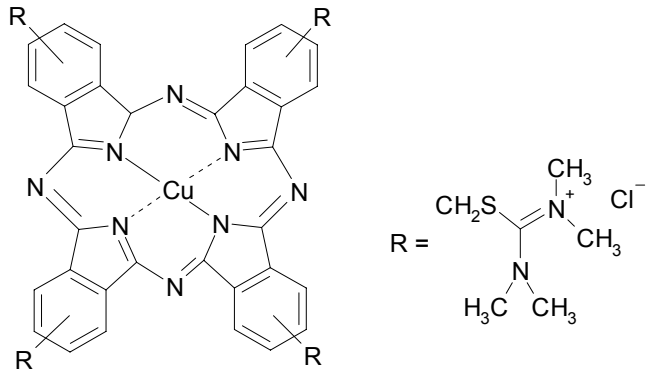



**Figure 18: Structure of Alcian Blue (Fluka)**

The use of Alcian Blue as a fixative necessitates additional considerations in molecular weight analyses by a sensitivity enhanced PAGE. Alcian Blue will not fix tetrasaccharides of sulphated glucoseaminoglucans, or fragments of polysaccharides containing less than 8 disaccharides units in a 10% polyacrylamide gel (Min and Cowman, 1986). In $H_2O$, Alcian Blue is precipitated by the buffer salts which leach from the gel. The solution may thus only be used once (Min and Cowman, 1986). The assay is linear between 0 and 100 mg/ml (Ramus, 1977).

## 2.3.3 Colorimetric Tests

There are various colorimetric tests that have been evaluated for a long period of time to detect sugars or their reducing ends. In this project four of these methods were used and adapted to the emerging questions. In the following the principles of the different tests are described. For every test, it is necessary to run a standard with the unknown.

### 2.3.3.1 Dubois Test (Dubois et al., 1956)

The colour development in the Dubois test is due to the conversion of the sugars into furfural derivates. The formation of furfural (see Figure 19) is an acid catalysed condensation reaction, separating 3 moles water from 1 mole hexose or pentose. The furfural product is condensed to a coloured compound through a phenolic compound (Newth, 1951). This can be detected at 490 nm. Colour formation is stable for several

hours and proportional to the amount of sugar present (Dubois et al., 1956). The detection limit is about 7 µg/l.

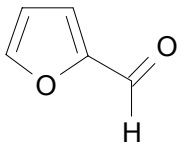



**Figure 19: Furfural**

### 2.3.3.2 Miller Test (Miller, 1959)

The Miller test is based on a redox reaction wherein the sugar is oxidised. If the medium contains too much of dextrose or other mono sugars, the test cannot be applied anymore. The absorbance values do not give an absolute amount of reducing sugars, but have to be calibrated with, for instance, fucose. Optimally, fucose is analysed as internal standard with every sample. Figure 20 shows the reaction scheme of the Miller test.

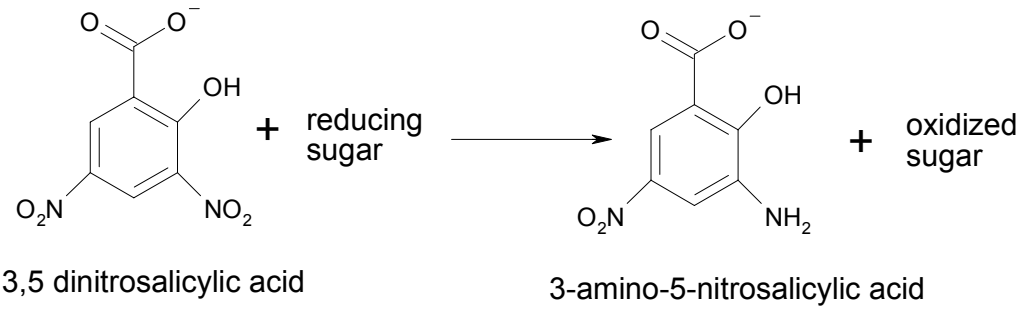



**Figure 20: Reaction scheme of the Miller test**

### 2.3.3.3 Somogyi-Nelson Test (Somogyi, 1952)

The method of Somogyi and Nelson is also based on a redox reaction. The reducing sugars are oxidised with a weak alkaline copper reagent to a sugar acid, while Cu(II) ions that are present in the solution, are reduced to Cu(I) ions. In the next step an arsenic molybdate reagent is used to oxidise these Cu(I) ions back to Cu(II) ions. The arsenic molybdate is thereby reduced, producing a characteristic blue colour that can be measured with a spectrophotometer (Nelson, 1944; Somogyi, 1952).

#### 2.3.3.4 Dische Test (Dische and Shettles, 1948)

The Dische test is a specific sugar oxidation reaction for the detection of fucose (methylpentoses). When used in combination with other colorimetric tests, the proportion of fucose -in comparison to other sugars- can be detected. The principle of this test is based on the relative absorption measured between two wavelengths, as different sugars produce different colours. The detection limit of this method is 10 µg/l. The phenolic reagent is carbazole (dibenzopyrrole).

### 2.3.4 Elemental Analysis

Elemental analysis is a form of combustion analysis. The analysed substances are burned completely and the quantitative measurements of the oxidation products is linked to the composition of the original molecules. The percentage of different atoms in the molecule can be determined.

### 2.3.5 GC/MS (Gas Chromatography Mass Spectrometry)

By gas chromatography all vaporisable (organic) substances can be separated. Common mobile phases are helium, argon and nitrogen. A very thin tube is used as a column which is filled with the solid stationary phase, e.g. silica gel. Samples are injected as fluids into the gas flow using a syringe. Due to different characteristics in absorption and solubility the substances migrate with different velocities through the stationary phase. At the end of the column, there has to be a detection system (mostly thermal conductivity detector) that measures the leaving substances. Polysaccharides cannot be measured directly this way. They have to be hydrolysed first in order to produce monosaccharides. These smaller molecules have then to be silylated to form thermo stable ethers which can then be analysed by gas chromatography. In our case, a mass spectrometry system is added to the GC separation. It creates charged particles (ions) from the eluted molecules. The ions are analysed to provide information about the molecular weight of the compound. The mass of the fragment divided by the charge is the mass to charge ratio (m/z). Since most fragments have a charge of +1, the m/z represents the molecular weight of the fragment.

### 2.3.6 IEC (Ion Exchange Chromatography)

Ion Exchange Chromatography also belongs to the liquid separation systems. Separation of the sample molecules results from their charge. In this case, the anionic polysaccharides bind to the positively charged ligands attached to the filling material of the column. Using

increasing ionic strength (salt gradient) or a pH shift, the different parts of the sample can be eluted. For instance, the matrix consists of a special cellulose, DEAE (diethylaminoethyl)-cellulose, which is shown in Figure 21.

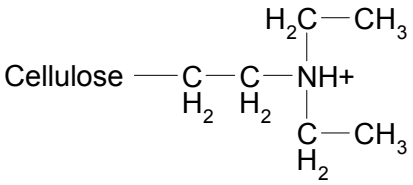



**Figure 21: DEAE-Cellulose (Mulloy et al., 1994; Pereira et al., 1999)**

## 2.4 Background Information for Bioactivity Tests

### 2.4.1 Coagulation Cascade

Blood coagulation is a very complex part of the haemostasis (the cessation of blood loss from a damaged vessel). A high number of different factors are involved in blood coagulation. All these factors are specially regulated. Anti-coagulant compounds are desperately needed, as damages (for example through genetic modifications) can occur in every factor of this complex cascade. The most famous abnormality is the malfunction of factor VIII leading to the well known haemophilia disease. Potent non-toxic anti-coagulant compounds are thus of great interest. The coagulation cascade can be divided into three pathways. The contact activation pathway (intrinsic pathway) and the tissue factor pathway (extrinsic pathway) that lead to fibrin formation. The damaged blood vessel is covered by a platelet and fibrin-containing clot to stop bleeding. These two pathways both activate the "final common pathway" of Factor X, thrombin and fibrin. Figure 22 shows both the intrinsic and the extrinsic pathway. The active coagulation factors are generally serine proteases (enzymes) and are shown in red. There are some exceptions. For example, FVIII and FV are glycoproteins and Factor XIII is a transglutaminase. Red arrows symbolize the proteolytic activation of other factors in the cascade. Helping factors such as $Ca^{2+}$ and the phospholipid membrane are marked in green. The names of the coagulation factors are shown in Table 5.

**Table 5: Human blood coagulation factors (Voet and Voet, 1994)**

| Factor | Name | Molecular weight of the subunit in kDa |
|---|---|---|
| I | Fibrinogen | 340 |
| II | Prothrombin | 72 |
| II | Tissue-Thromboplastin | 37 |
| IV | $Ca^{2+}$ | |
| $V_a$ | Proaccelerin | 330 |
| VII | Proconvertin | 50 |
| VIII | Antihaemophilic Factor | 330 |
| IX | Christmas-Factor | 56 |
| X | Stuart-Factor | 56 |
| XI | Plasma Thromboplastin Antecedent (PTA) | 160 |
| XII | Hageman-Factor | 80 |
| XIII | Fibrin stabilising Factor (FSF) | 320 |
| | Präcallicrein | 88 |
| | High molecular Kininogen | 150 |

[a] Factor $V_a$ was named Factor VI previously. Thus Factor VI is missing in the table

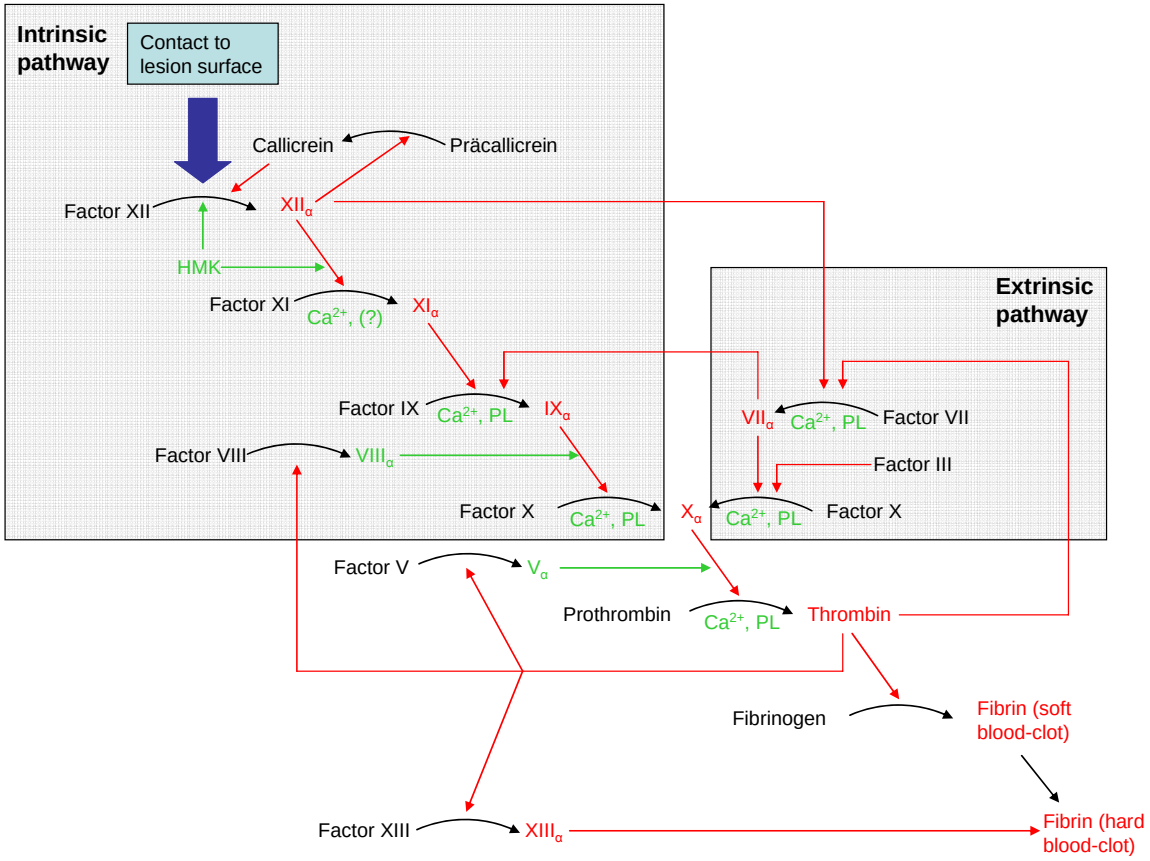



**Figure 22: Coagulation cascade modified after Voet (Voet and Voet, 1994); red arrows symbolise the proteolytic activation of other factors in the cascade. Helping factors are marked in green.**

### 2.4.2 HCMV (Human Cytomegalo Virus)

The human cytomegalovirus, like the HHV-6 and the HHV-7, belongs to the family of β-herpes-viruses. It is only partly known how the virus attaches itself to the host cell. It is very difficult to identify cellular receptors, since HCMV is able to infect various cell types (Jarvis and Nelson, 2002). As HCMV is able to infect such a high number of different cells it is suggested that HCMV can use several receptors or/and common cell receptors for infection (Compton, 2004). Several virus infections can be inhibited by various sulphated polysaccharides such as carrageenans (Adhikari et al., 2006; Carlucci et al., 1997a; Gonzalez et al., 1987), sulphated galactans (Carlucci et al., 1997b), and fucoidans (Lapshina et al., 2006; Lapshina et al., 2007; Lee et al., 2004a; Lee et al., 2004b; Ponce et al., 2003; Witvrouw and De Clercq, 1997). The first attempts concerning the activity of fucoidan from *Fucus vesiculosus* against human immunodeficiency virus (HIV) were done about 20 years ago (Baba et al., 1988; Beress et al., 1993).

**Synthetic Virus Statica against Herpes Virus Infections**

There is a large number of synthetically produced compounds that can be used against virus infection. Many of those compounds such as acyclovir and penciclovir show a very affective and reliable effect against virus infection, whereas other compounds such as ganciclovir (see Figure 23) also display toxic affects against the cell as well (Villarreal, 2001).

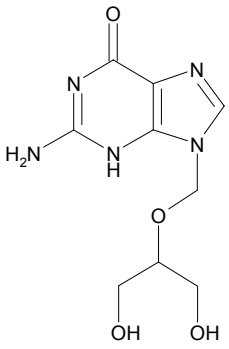



**Figure 23: Ganciclovir, e.g in Cymeven®, Roche**

### 2.4.3 *In vivo* Two-Stage Carcinogenesis Test on Mouse Skin Papillomas Induced by DMBA/TPA or Peroxinitrite/TPA

The *in vivo* two-stage carcinogenesis test is performed with mice. It is measured, how the skin cancer development is inhibited by several polysaccharides compared to the positive control. Cancer initiation takes place with dimethylbenz(α)anthracene (DMBA; 390 nmol)

or peroxinitrite (NO; 35 µg) in acetone (0.1 ml). One week after this initial treatment the mice are tumour-promoted with the application of TPA (1.7 nmol) in acetone. The mice are treated either with the test compound or with acetone (control group) before this treatment. The incidence of papillomas in the control group and test substance group are observed weekly for 20 weeks. Figure 24 shows the chemical structures of the tumour-initiating compounds dimethylbenz(α)anthracene (DMBA) and peroxynitrite (NO) as well as the tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA).

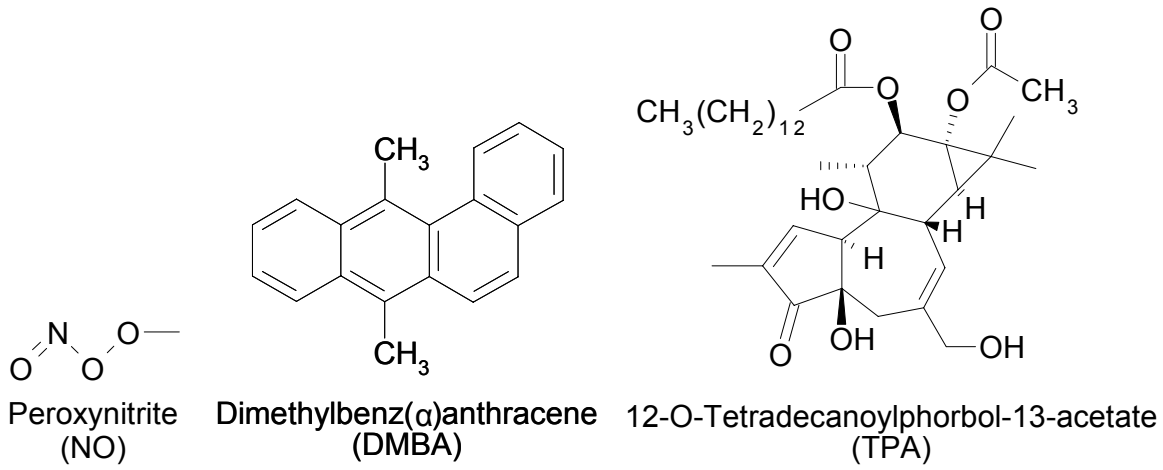



**Figure 24: Chemical structures of NO, DMBA and TPA**

### 2.4.4 Short Term *in vitro* Bioassay for the Inhibition of Epstein-Barr Virus Early Antigen (EBV-EA) Activation Induced by TPA

A second anti-tumour test is performed *in vitro* on Raji-cells (EBV genome carrying human lymphoblastoid cells, non producer type). With these cells, the inhibition of the TPA-induced activation of the Epstein-Barr virus (EBV) early antigen can be detected by indirect immuno fluorescence measurements. Raji cells are incubated in a medium containing n-butyric acid as an inducer, TPA and a known amount of the test compound (Langer et al., 2006). Activated cells can be stained with high titre EBV-EA-positive sera from nasopharyngeal carcinoma patients. Detection is later on performed with an indirect immuno fluorescence technique. Viability of the cells is assayed by Trypan Blue staining (Colombo et al., 2000; Fukuda et al., 2005; Ito et al., 2005; Sakurai et al., 2005; Tanaka et al., 2004). An evaluation of the EBV-EA tumour inhibition is often used as a primary screening for *in vitro* anti-tumour promoting activities (Colombo, 2005).

### 2.4.5 Antibody Selection Process via Phage Display ("Panning")

Antibodies can be used to select specific clones from a large library of phage displaying many different determinants. The phage are thus 'antibody-selectable'(Parmley and Smith, 1988). The phage can be modified genetically to produce different foreign peptides or proteins and present them on their surface (Smith, 1985; Winter and Milstein, 1991). The structure of the foreign protein as well as the phage envelope remain in their functional configuration (Barbas III et al., 1991; Li et al., 2002).

The target molecules (here fucoidan) are immobilised on surfaces such as polystyrole micro titre plates. For selection, an antibody phage library containing several scFv-phage presenting different target proteins on their surfaces is needed, e.g. a combination of the libraries HAL4 and 7 (Hust et al., 2007). Upon incubation with scFv-phage from an antibody phage-display-library specific scFv-phage will bind if present in the library. Unspecific bound phage can be washed away. The "binders" can be eluted and used for infection of *E. coli* cells for amplification of scFv-phage. For infection the phagemids are packed into the outer envelope of M 13 phage. This helper phage provides the information for the envelope proteins to produce phage particles. An alternative helper phage is the hyper phage. This phage can present proteins polyvalent on its surface (Rondot et al., 2001). Subsequently the production of scFv-presenting phage for the selection process can be redone. After every "panning-round" the selection procedure gets harsher and more stringent. Three to five panning rounds have been shown to be sufficient to obtain a strong enrichment of specific binders (Breitling and Dübel, 1997).

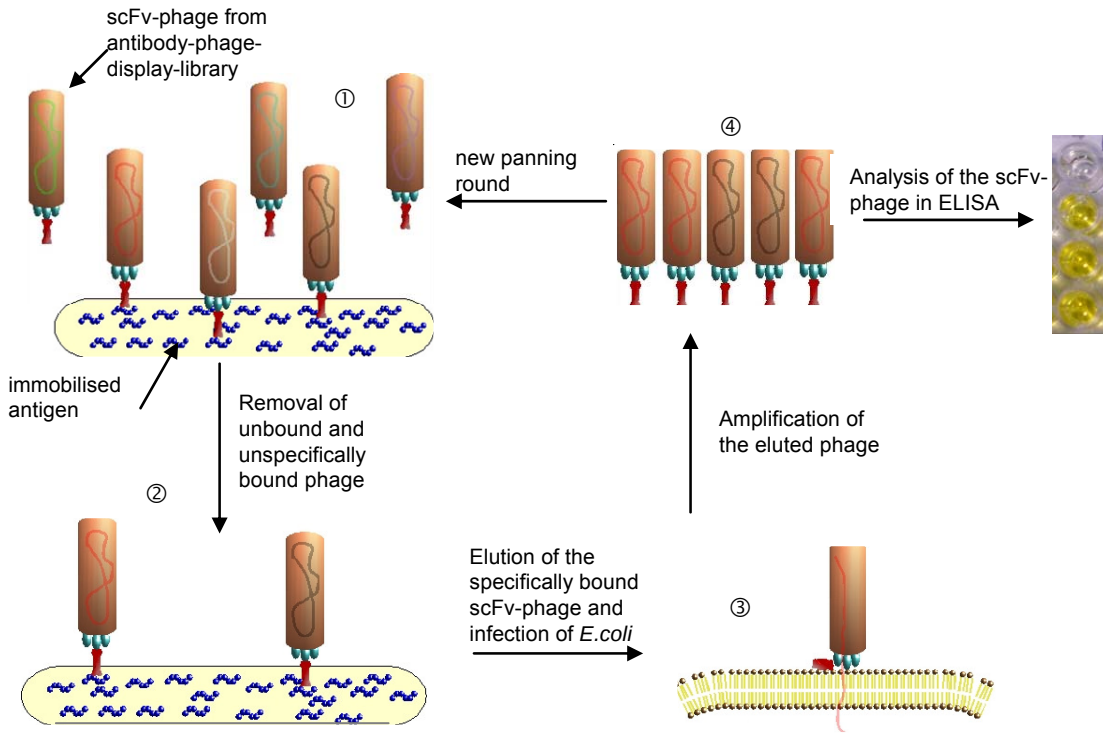



**Figure 25: Schematic panning scheme modified after Hust (Hust et al., 2007)**

The panning procedure (Figure 25) starts with scFv-phage from an antibody phage-display-library. The scFv-phage bind to the immobilised antigens and the unspecifically bound phage are washed away. The specifically bound phage are used for infection of *E.coli* cells and are then amplified. Afterwards these amplified phage are used in new panning rounds with harsher conditions at each time.

# 3 Materials and Methods

## 3.1 Sulphated Polysaccharides/ Oligosaccharides

### 3.1.1 Sources of Fucoidan

The first task of this project was to produce a standardised fucoidan. It was possible to purchase fucoidan in 5g amounts from Sigma (250 €). This fucoidan was analysed and showed a high amount of impurities (about 25%) with inorganic substances. Fucoidan had a brownish colour and its appearance varied between different batches. It supervened that it was quite expensive. Because of these factors, extracting one's own fucoidan from natural sources seemed an attractive alternative. By controlling the collection and extraction processes, one can be sure to obtain the same quality each time and -if the composition of the material changes- the different factors are known and can be compensated for. In addition, it is cheaper to produce large amounts.

In April 2005, August 2006, and July 2007, large amounts of the brown algae *Fucus vesiculosus* were collected at Wilhelmshaven Südstrand (N 53°51', E 8°14') during incipient low tide. The thalli were washed and dried at ambient conditions. Thereafter they were dried at 80 °C in an oven to prevent moulding. The material was then mortared for further use. Sigma Fucoidan was used as purchased.

### 3.1.2 Extraction and Processing of Fucoidan from Brown Algae *Fucus vesiculosus*

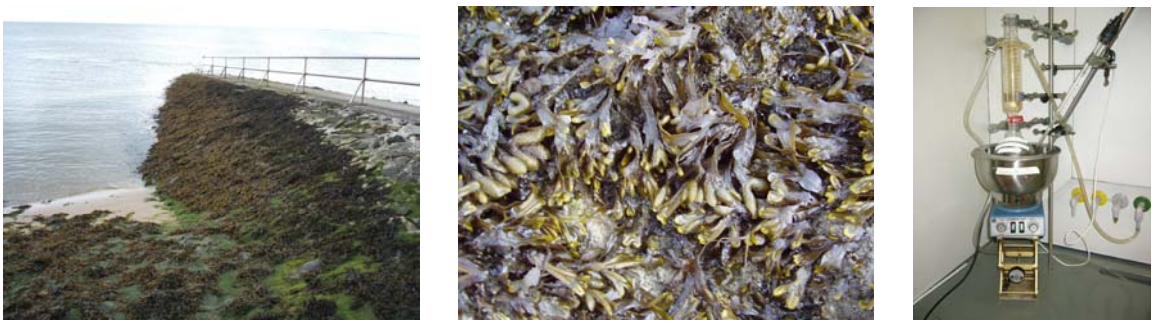
Different extraction procedures were tested both in our lab as well as in the lab of our cooperation partner (TU Kaiserslautern). The best procedure with regard to yield and quality for our application is presented in the following: Before extraction, 50g of the dried algal material was soaked with a mixture of ethanol:formaldehyde:$H_2O$ dest. (80:5:15) over night to flush out chlorophylls and other colourings. This mixture was then decanted and the remaining algal material was defatted with acetone.

Extraction was carried out twice in a Soxleth apparatus at 70 °C in a 0.1 M HCl solution, containing 4% $CaCl_2$ in water. After extraction the supernatant was collected and cooled to remove alginates by precipitation. The supernatant was neutralised with ammonium carbonate ($(NH_4)_2CO_3$). Residual polysaccharides were then precipitated with a 2.5 times amount of pure ethanol. At this stage, the solution can be kept over night to let the polysaccharides precipitate properly. After precipitation the ethanol was decanted. The

moist polysaccharides were centrifuged at 10,000 $min^{-1}$ for about 15 min in a Suprafuge 22 (Heraeus; HFA 14290) and the rest of the ethanol was discarded. The pellet was then dissolved into a small amount of desalted water and freeze dried at -60 °C and < 0,5 mbar in a freeze drier (Laborgefriertrockner ALPHA 1-4 LSC, Christ).

After freeze drying different extraction fractions were pooled, resolved in water, and ultrafiltrated through a 100 kDa ultrafiltration membrane (Vivaflow 200, Sartorius Göttingen). Both retentate and permeate were collected and freeze dried again. The two fractions were then called FVEhigh (*Fucus vesiculosus* extracts of high molecular weight; >100kDa) and FVElow (*Fucus vesiculosus* extracts of low molecular weight; <100 kDa). These polysaccharides were then used for further analysis. If necessary, the fractions were dialysed (Visking® dialysis tubing, regenerated cellulose; 12-14 kDa, pore diameter 25 Å) before the final lyophilisation process.

To evaluate the purity and distribution of both fractions, the raw extraction material (before ultrafiltration) and the two fractions (after ultrafiltration) were analysed in a SE-HPLC-system. The system was developed during this project and consisted of two PL aquagel OH-mixed 8 µm columns that were successively connected to each other. The detection was carried out by a refractive index detector from Shodex (Shodex RI 101). Former measurements also included UV detection. This was only possible with fucoidan purchased from Sigma, owing to its brownish colour. The self-produced material was white (FVEhigh) or very slightly yellow (FVElow) and did not give a strong signal in UV detection. Figure 26 shows the collection point of the algae at the coast of Wilhelmshaven (A), the algae *Fucus vesiculosus* (B) and the extraction apparatus (C).



A B C

**Figure 26: A = Collection point in Wilhelmshaven Südstrand (N 53°51', E 8°14'); B = *Fucus vesiculosus*; C = Extraction apparatus**

### 3.1.3 Extraction and Processing of Fucoidan from Brown Algae *Laminaria digitata*

Extraction and processing of the fucoidan from *Laminaria digitata* was done according to the extraction procedure of fucoidan from *Fucus vesiculosus*. *Laminaria digitata* was purchased as dry powder from MaBitec® GmbH; Bruckmühl, so the first drying steps were omitted. The smaller fractions of LDE (*Laminaria digitata* extracts) after Ultrafiltration were not collected, so only LDEhigh was produced.

### 3.1.4 C-PAGE (Carbohydrate Polyacrylamide Gel Electrophoresis)

The carbohydrate polyacrylamide gel electrophoresis was established after the description of Descamps (Descamps et al., 2006). A normal PAGE was done at 27% density of the running gel. The stacking gel had a density of 6%. The conversion to a bigger gel system was developed in this institute throughout the project. As a gel stand a former DNA sequencer system (T-REX Sequencer, OWL) was used. Spacers are custom-made to 1 mm thickness and 450 mm length of Teflon. The samples were diluted into a 10% (w/v) sucrose solution with phenol red (¼ sample, ¾ sucrose). About 2 to 5 µl were applied to the gel. The 20x buffer diluted to 1x can be used as migration buffer. The gel was run at 200 V until the phenol red of the loading buffer was at ¾ of the gel. The exact recipes for the gels are shown in Table 6 and Table 7.

**Table 6: Stacking gel (6%)**

| Chemical | Amount for 1 mini gel | Amount for 1 big gel |
|---|---|---|
| Acryl amide | 0.333 ml | 0.3 ml |
| Buffer (20x) | 0.1 ml | 0.2 ml |
| $H_2O$ dest. | 1.56 ml | 1 ml |
| APS | 33.3 µl | 15 µl |
| TEMED | 1.33 µl | 2 µl |

**Table 7: Running gel (27%)**

| Chemical | Amount for 1 mini gel | Amount for 1 big gel |
|---|---|---|
| Acryl amide | 3 ml | 99 ml |
| Buffer (20x) | 0.2 ml | 11 ml |
| $H_2O$ dest. | 0.6 ml | - |
| APS | 26.6 µl | 1.1 ml |
| TEMED | 2.66 µl | 55 µl |

**Staining Procedure**

The staining of the gel can be done either directly after running the gel or after storing it in buffer-soaked kitchen paper overnight. The staining procedure was adapted from Descamps (Descamps et al., 2006). It was a combined staining with Alcian Blue (Sigma) (Cowman et al., 1984) and silver nitrate. First the gel was stained in 0.5% (w/v) Alcian Blue in water for 10 min under agitation. It was optimal to prepare fresh solution every time, but as long as the solution had not precipitated, it could be reused for a few days. Destaining of the Alcian Blue was done with water under agitation until the gel turned clear again (sometimes an overnight washing step was necessary). When the gel was clear, it was stained with a 0.4% (w/v) silver nitrate solution in the dark for 10 min without agitation. The silver nitrate solution had to be freshly prepared and kept in the dark. Three washing steps of about 1 min in $H_2O$ under agitation followed the silver nitrate staining step. The development reaction took place by adding a 7% (w/v) sodium carbonate solution under manual agitation. By adding small amounts (20 µl) of formaldehyde and manually agitating the gel, the reaction solutions mixed and bands appeared. To stop the reaction a 5% (v/v) acetic acid solution was added.

Documentation was done with gel documentation software and/or ordinary digital cameras. It was important to document both the Alcian Blue staining and the combined staining, as differences could occur.

### 3.1.5 TLC–Analysis (Thin Layer Chromatography)

TLC is a potent method to detect various low-molecular-weight organic substances. These are dissolved in suitable solvents and applied to the TLC plate. In this work, sugars and fungal metabolites were detected. The sugars were dissolved in water and 3-5 µl of this solution were applied to the TLC plate around 1-2 cm from the bottom with a Hamilton syringe. The different solvents for the fungal metabolites are stated in the respective chapters. The application spots were dried with a blow-dryer to avoid interference with the water. The TLC plates were then put into a lockable container (development chamber) which had been filled with 1-2 cm layer of the mobile phase around 10 min prior for saturation of the chamber. The sides of the TLC plate were not allowed to touch the sides of the development chamber. When the mobile phase had nearly reached the top of the TLC plate (0.5 cm from the edge), chromatography was finished and the TLC plate was removed from the chamber. After drying different visualisation steps could be applied which are stated in the respective chapters of this thesis.

To compare the results, the so called $R_F$-value was determined for each substance. It was calculated as ratio of the distance covered by the sample component to that covered by the solvent. Due to that, a $R_F$-value is always between 0 and 1. This correlation is also shown in Equation 1. Figure 27 shows the general principle of the TLC analysis.

$$R_F\text{-value [-]} = \frac{\text{Distance covered by the compound to be analysed [cm]}}{\text{Distance covered by the solvent [cm]}}$$

**Equation 1: Calculation of the $R_F$-value**

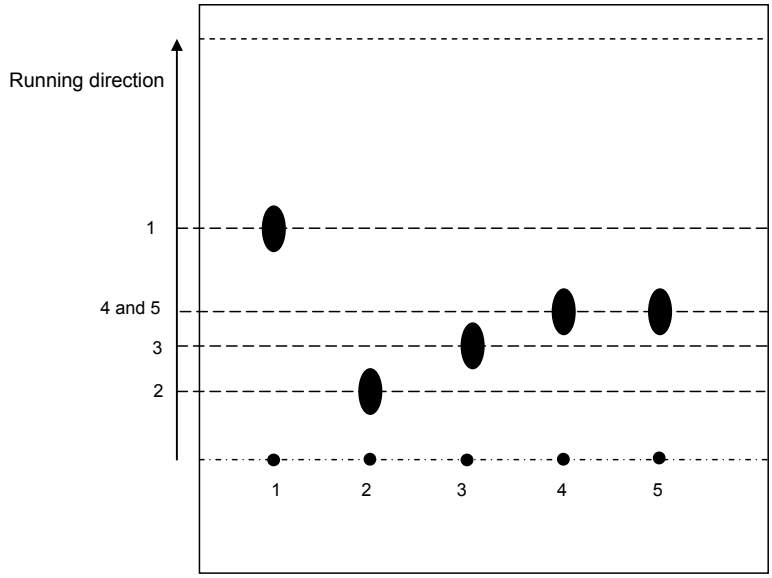



**Figure 27: Principle of TLC-analysis**

### 3.1.6 SE-HPLC (Size Exclusion High Performance Liquid Chromatography)

Size exclusion chromatography is a very potent analysis system for large polysaccharides. It is widely used in the determination of polysaccharides, particularly for dextrans (Fleury and Lahaye, 1993; Geresh et al., 2002; Granath and Flodin, 1961; Theisen et al., 2000; Yuan and Song, 2005). The system could be used for fucoidans as well. Considering that fucoidan is a charged molecule, the solvent had to be chosen so that the fucoidans move constantly in the column. As no system existed in the laboratory when the project was started, this method had to be established first. Parameters for the new method are shown in Table 8.

In literature the combination of varying viscosity and errors in the experimental determination of retention volume easily introduced a total error of 15% on the detected

molecular weight of the sulphated polysaccharide κ-carrageenan. The use of an universal calibration method for an exact molecular weight determination of anionic natural polymers is therefore still questionable (Malfait et al., 1990).

**Table 8: Parameters for the SE-HPLC measurements**

| Parameter | Characteristic |
|---|---|
| Column | Two PL (Polymer lab)aquagel-OH-mixed; 8 µm<br>Rigid macroporous hydrophilic polymer |
| Solvent flow rate | 1 ml/min |
| Injection volume | 100 µl |
| Solvent | $LiNO_3$ (0.01 M) in MilliQ water |
| Detection | Refractive index detector |
| Software | Clarity Extensions GPC<br>© DataApex Ltd. 2006<br>Podshradská 1<br>15500 Prague 5<br>The Czech Republic |

## 3.1.7 Colorimetric Tests

During this project several colorimetric (chemical) tests were used to determine the degradation of the FVEs.

### 3.1.7.1 Dubois Test

This test was adapted from Dubois (Dubois et al., 1956) and scaled to the specifications of this project. 200 µl of the sample and 200 µl of a 5% (w/v) phenol-solution in deionised water were filled into a 1.5 ml reaction vial and mixed carefully. Then 1 ml of concentrated sulphuric acid was added (this step had to be done very carefully as the sulphuric acid may spatter and cause severe injuries). After 10 min the samples were mixed vigorously for about 15 sec. 30 min later, the absorption could be measured at 490 nm in a spectrophotometer. Plastic cuvettes endured the acid if handled with care. As standard, different concentrations of a glucose solution were measured.

### 3.1.7.2 Miller (DNS) Test

This test was adapted from Miller (Miller, 1959) and scaled to the specifications of this project. At first the samples had to be defrosted, mixed intensively and centrifuged at 13,000 $min^{-1}$ for 5 min (Centrifuge 5810 R, Eppendorf). Subsequently 100 µl of the sample

and 100 µl of the DNS-reagent (see appendix for further information) were mixed in a 1.5 ml reaction vial. To avoid losses the mixture was centrifuged shortly. The samples were then heated (100 °C) in a water bath and the reaction was stopped in ice water after 7 min. When the samples had cooled, 800 ml of deionised water was added and they were measured at 540 nm in a spectrophotometer using plastic cuvettes. As standard solutions different amounts of L-fucose in water were used.

#### 3.1.7.3 Somogyi-Nelson Test

This test was adapted from Somogyi (Somogyi, 1952) and Nelson (Nelson, 1944) and scaled to the specifications of this project. The reagents used are described in the appendix. 500 µl of the sample was diluted an equal volume with water. After dilution 1 ml of copper reagent was added, mixed carefully and incubated for 10 min in boiling water. The mixture was then cooled on ice for approximately 5 min to stop the reaction. Afterwards, 1 ml of the arsenic molybdate reagent was added and mixed carefully. Subsequently 3 ml of water was added and mixed carefully. After 15 min incubation at room temperature the mixture could be analysed at 500 nm in a spectrophotometer. As standard different concentrations of glucose and L-fucose in water were used.

#### 3.1.7.4 Dische Test

For the detection of L-fucose 400 µl of the sample were mixed with 1.8 ml concentrated sulphuric acid (1:6). The mixture was subsequently cooked in a water bath for 10 min and the reaction was stopped by cooling on ice. Thereafter 40 µl of a 3% (w/v) L-Cystein-Hydrochloride solution was added. The absorption was measured at 396 nm and 430 nm. With the difference of these two measurements the influence of other sugars could be neglected. As a standard L-fucose solutions were used in the concentrations of 0.01 g/l, 0.02 g/l, 0.04 g/l, 0.06 g/l, 0.1 g/l, and 0.2 g/l.

### 3.1.8 Fucoidan Degradation Quick Test (Kitamikado et al., 1990)

A fucoidan degradation quick test was adapted to the simple turbidimetric method to detect alginate degradation of Kitamikado (Kitamikado et al., 1990). The microorganisms were grown in fucoidan- or extract-containing media. The medium used included 0.5% peptone, 0.1% yeast extract, 3% NaCl for marine bacteria or 0.5% NaCl for other bacteria as well as 0.1% sodium alginate. The media were adjusted to pH 7.6 (marine bacteria) or 7.2 with a

NaOH solution and sterilised by heating at 120 °C for 15 min. Bacteria samples were inoculated into 5 ml medium after cooling and incubated for 1 to 7 days at 25 °C. On each day of incubation 0.5 ml of the culture were withdrawn with a sterile glass tube and centrifuged at 3,000 $min^{-1}$ for 10 min (Centrifuge 5810 R, Eppendorf). 200 µl of the supernatant were poured into a small Pyrex test tube and 2.0 ml of acid albumin solution was added. (Acid albumin solution: 3.26 g of sodium acetate, 4.56 ml of glacial acetic acid, 1.0 g of BSA (bovine serum albumin) were dissolved in 1 l of distilled water; pH adjusted to 3.72 to 3.78 with HCl). A Fucoidan Sigma- or extract-degrading culture was detected by the transparency or by significant decrease in turbidity of the test solution.

### 3.1.9 Elemental Analysis

For elemental analysis the samples were sent to Ilse Beetz, Mikroanalytisches Laboratorium, Industriestraße 10, 96317 Kronach. Analysis was performed on S, C, O, and H. Additionally, the amount of inorganic compounds was measured as ashes.

### 3.1.10 GC/MS (Gas Chromatography Mass Spectrometry)

The analyses were performed in the Helmholtz Centre for Infection Biology (HZI) in Braunschweig by Dr. Manfred Nimtz to elucidate the monosaccharide composition of the heterogeneous polysaccharides. To determine the monosaccharide composition, the polysaccharides had to be degraded first to their monosaccharides which were then silylated, to facilitate their analysis by gas chromatography (capillary gas chromatograph, HRGC 5160 mega series, Carlo Erba/Hofheim/TS). The system had to be calibrated with equally treated, known monosaccharides. The selection of standards should include the possible monosaccharides usually found in brown algae. The problem with this system is the adjustment of the degradation process. It was very difficult to distinguish between total degradation and loss of special monosaccharides, e.g. fucose. Several measurements had to be done to get an indication of the composition. After the measurement in the GC, the now separated monosaccharides (seen as peak signals) were measured in a quadruple-mass spectrometer (MAT 4515 Finnigan/USA). This machine also had to be calibrated with known sugars prior to the experiment.

**Accomplishment**

Methanolic HCl (0.625 M) was produced by dropping 4.4 ml acetyl chloride into dry ice-cold methanol (stirring and cooling). As silylation reagent Bis(trimethylsilyl)-tri fluor acetamid (BSTFA) with 1% of tri methyl chlorsilan (700 µl BSTFA + 7 µl tri methyl chlorsilan) was used. "Wheaton vials" with a Teflon gasket were used as reaction containers. When talking about droplets, the drop size of Pasteur pipettes was applied as standard. Meso-inositol (100 ng to 1 µg) was used as an internal standard. First, the Oligosaccharides were hydrolysed by adding 200 µl (15 drops) of methanolic HCl and 50 µl (5 drops) methyl acetate (acetic acid methyl ether) to the dried sample. After successful mixing the solution hydrolysed for 16h (over night) at 70 °C. Afterwards 50 µl (5 drops) tert.-butanol were added and the solvent was evaporated with nitrogen. 100 µl (8 drops) pyridine and 50 µl (5 drops) trimethylsilylation reagent were added and heated for 1h at 70 °C (cabinet dryer). After cooling down, one part of the sample was diluted with two parts of cyclohexane and filled into special GC/MS vials (tapered insert). When fucose was anticipated in the sample no nitrogen blowoff took place (fugitive sugar derivatives like fucose may be blown away). The sample was then diluted with 100 µl cyclohexane and injected into the GC (Chaplin, 1982; Chaplin, 1986). Table 9 gives the important parameters of the HZI-measurements.

**Table 9: Parameters of GC/MS-measurements performed at the HZI, Braunschweig**

| Parameter | Characteristic |
|---|---|
| Gas chromatograph | HRGC 5160 mega series, Carlo Erba/ Hofheim/TS |
| Capillary column | DB 5 with 0.1 µm of film thickness, 30 m length and 0.32 outer diameter |
| Carrier gas | Helium |
| Mass spectrometer | MAT 4515 Finnigan/USA |
| Ionisation method | Electron Impact (EI) |

### 3.1.11 IEC (Ion Exchange Chromatography)

As fucoidans are charged molecules, ion exchange chromatography was used to analyse the polysaccharides. The parameters used can be seen in Table 10. Fractions were collected (Fraction collector SF 2100 W; Pharmacia) and analysed by the colorimetric methods stated in chapter 3.1.7.

**Table 10: Parameters of IEC-measurements**

| Parameter | Characteristic |
|---|---|
| Filling material | DEAE cellulose, Servacell 23 SS; Serva |
| Flow rate | Gradient flow (linear from 0M to 2M NaCl); 0.16 ml/min (controller LCC 501 Plus; Pharmacia) |
| Temperature | Room temperature |
| Columns | C 10/40 and C 16/20 (Pharmacia) |
| Solvent | 50 mM Tris-HCl buffer; pH 7.0 |
| Sample injection volume | 1 ml (conc. 10g/l) |

As analysis of fucoidans with this method were very time consuming (12 h for one measurement) and required very high concentrations of the fucoidan sample (10g/l), the ion exchange chromatography was abandoned as the method of choice. It is merely mentioned in this thesis for the sake of completeness.

### 3.1.12 Modification Experiments

To produce oligosaccharides of the FVEs different attempts were used. This included physical, chemical, and enzymatic degradation methods.

#### 3.1.12.1 Physical degradation by Ultrasonic Forces

These experiments were performed with assistance of Ingo Kampen and Stefanie Michel (IPAT, Institut für Partikeltechnik, TU Braunschweig). Degradation experiments were conducted with the polysaccharide samples at a concentration of 1 g/l solutions in 0.01 M $LiNO_3$. The samples were cooled in an ice bath throughout the experiment and kept in the refrigerator when the temperature exceeded 55 °C. It was very important to use small plastic beakers as glass may burst under the sonication. The sonotrode had to be submerged at least 1.5 cm beneath the fluid surface. After sampling the height of the sonotrode had to be adjusted.

Two different Watt strengths were used in the experiments; 150 and 300 Watts. The sonotrode was a Labsonic L from Braun Melsungen. The acoustic irradiation sequence was pulsed to 5 sec of acoustic irradiation and 10 sec of recreation. This was done to keep the temperature increase to a minimum. When the temperature increase seemed too steep, the mixture was cooled in a refrigerator for 30 min before continuing the experiment. With Equation 2 the application of energy per g sugar can be calculated.

$$E_{spec} = \frac{P \cdot t}{V_{susp} \cdot C_{sugar}}$$

**Equation 2: $E_{spec}$ = Specific energy application on the polysaccharide [$m^2 \cdot s^{-2}$]; P = power [Watt = $J \cdot s^{-1}$ = $kg \cdot m^2 \cdot s^{-3}$]; t = time [s]; $V_{susp}$ = volume of the suspension [$m^3$]; $c_{sugar}$ = concentration of the sugar [$kg \cdot m^{-3}$]**

Detection was performed on TLC plates (silicagel 60 $F_{254}$, Merck). 3 to 5 µl (different attempts were performed) of the samples taken during degradation were spotted on the TLC plate and dried with hot air. For the development the TLC plate was placed into a development chamber with n-butanol : ethanol : distilled water (30:50:20) as mobile phase. After development the TLC plate was dried and then sprayed with anisaldehyde : sulphuric acid (97%) : glacial acetic acid (0.5:1:50) to detect the mono-, oligo- and polysaccharides produced. For comparison 5 g/l solutions of different mono sugars, polysaccharides, and pure 0.01 M $LiNO_3$ were applied.

#### 3.1.12.2 Chemical Degradation by Acid Hydrolysis

To degrade the FVE polysaccharides, the method of Pomin (Pomin et al., 2005) was used. 10 mg of the respective FVE was dissolved in 1.25 ml of 0.01 M HCl and kept in a 60 °C water bath. To stop the reaction 1.25 ml of ice cold 0.01 M NaOH was added. After degradation, the hydrolysed FVEs were lyophilisied (Laborgefriertrockner ALPHA 1-4 LSC, Christ) and dialysed (Visking® dialysis tubing, regenerated cellulose; 12-14 kDa, pore diameter 25 Å) against deionised water before being lyophilised again. Afterwards the oligosaccharides could be characterised with various methods.

#### 3.1.12.3 Enzymatic Degradation by Polysaccharide Hydrolysing Enzymes

Another option for degrading heterogeneous polysaccharides is the enzymatical degradation. Different enzymes are able to cleave sulphated fucose components. It was not possible to purchase a fucoidanase; the only fucoidanase that has been detected thus far is patented by a Japanese group (Sakai et al., 1996) and it is not available to other researchers. In 2006 the genetic structure of a fucoidanase was published by Colin (Colin et al., 2006). So far no attempts have been performed to express this enzyme in a suitable host.

Other attempts have been made with supernatants or cell disruption solutions of different cultivated microorganisms (Zuccaro et al., 2004). These solutions are also called enzyme solutions and are thus being described in the enzymatic degrading experiments. As no suitable enzyme was available, enzymatic degradation was thus performed in this project as well. One has to distinguish between the cultivation experiments: The polysaccharides were given as (single) C-source and the degradation of the polysaccharides was detected as the growth ability on this polysaccharides and the enzymatic degradation: The supernatant or the cell content after cell disruption was used as an enzyme solution. These solutions were given to defined polysaccharide solutions.
The amount of enzyme solution and the molarities of the sugar solutions differed between the different experiments. Experiments with the commercially available enzymes (see chapter 3.3.11) were comparable to the enzymatic degradation experiments. Detection systems were also different according to the various products to be detected. The commercial enzyme products could be detected by photo spectrometer (96 well plates) as the substances produce colourful (p-nitrophenol-containing) end products.

## 3.2 Bioactivity Determination

For bioactivity determination cooperation with experienced partners was necessary. Four different bioactivities of the produced FVEs were tested.

### 3.2.1 FVEs, LDEs and Fucoidan Sigma as Anti-coagulants

This test was conducted in the department of molecular biology with the help of Dr. Franz Vauti. Tests were performed in a coagulometer (Amelung-Coagulometer KC 4A). Blood was taken with heparinated glass capillary (no. 563, Assistant, Sondheim, Germany) from mice by Dr. Franz Vauti (around 400 µl; behind the eye). The tested substances were dissolved in Hepato Quick citrate buffer, pH 7.45 in a concentration of 1 g/l. Several dilutions of these dissolved substances were tested. 100 µl of the diluted sample were mixed with 20 µl of the mouse blood. Then 200 µl of Hepato reagent were added and the mixture was incubated without agitation for 2 min at 37 °C. After incubation 100 µl 0.01 M calcium chloride was added to start coagulation and the chronometry started. Coagulation time was measured in sec. With increasing coagulation time, the substance is more potent as anti-coagulant.

### 3.2.2 Determination of Anti-viral Activity

The determination of antiviral activity was done in cooperation with Stefanie Thulke from the Charité-Universitätsmedizin Berlin, CCM – Medizinische Klinik m.S. Onkologie/Hämatologie, Charitéplatz 1, 10117 Berlin, Germany and Yvonne Naumann from the Institute of Bioprocess Engineering, Friedrich-Alexander University of Erlangen-Nuremburg. The accomplishment of the experiments was adapted to the description in the doctoral thesis of Tanja König, Friedrich-Alexander University of Erlangen-Nuremburg. Experiments were performed by Stefanie Thulke.

To test the strain of HCMV AD 169, $4x10^4$ MRC-5 cells were cultivated in 200 µl of cell culture medium in a 96 well plate for 24 hours. Temperature was adjusted to 37 °C and $CO_2$ content was 5%. After incubation the medium was removed and 50 µl of the fucoidan solutions as well as 50 µl of HCMV containing supernatant were added and incubated for another three days. Infection was caused with 1 to 4 x $10^{-3}$ infectious virus particles per cell (also called: Multiplicity of Infection, MOI). Five fucoidan concentrations as well as one blank sample were tested. The HCMV titre was checked by staining the viral delayed early antigens in infected cells by peroxidase.

Cells were washed twice with PBS and then fixed with methanol at -20 °C. These were then incubated with anti-HCMV p52 antibodies (CMV clone CCH2, Dako, Hamburg) and a peroxidase conjugated AffiniPure Goat-Anti-Mouse IgG (Dianova, Hamburg) for 30 min at 20 °C. Staining was performed with 50 µl 3-Amino-9-ethylcarbazole solution (AEC solution). This staining solution was prepared with one tablet (20 mg) of AEC (Sigma), dissolved in 4 ml N,N-Dimethylformamide (DMF, Merck), 16 ml of Na-Acetate (0.1 M, pH 5, Merck), and 40 µl of $H_2O_2$.

The virus titre was then determined by counting the stained cells. One stained cell was equivalent to one infected cell, corresponding to one infectious HCMV-particle (antigen producing unit, APU). To determine the virus inhibiting fucoidan concentration $IC_{50}$, which marks the reduction of virus infection to 50% the number of infected cells was plotted against fucoidan concentration.

### 3.2.3 Determination of Anti-tumoral Activity

The determination of anti-tumoral activity was done by Harukuni Tokuda from the Department of Biochemistry, Prefectural University of Medicine, Kyoto, Japan. Two different tests were performed in order to describe the anti-tumoral activity of the FVEs.

### 3.2.3.1 *In vivo* Two-Stage Carcinogenesis Test on Mouse Skin Papillomas Induced by DMBA/TPA or Peroxinitrite/TPA

For this test female ICR (Imprinting Control Region) mice, 5 to 6 weeks in age, were obtained from SLC Co. Ltd. (Shizuoka, Japan). The mice were grouped to 15 animals/group in polycarbonate cages. The back of the mice were shaved with surgical clippers before the first day of initiation. Initiation took place with dimethylbenz(α)anthracene (DMBA; 390 nmol) or peroxinitrite (NO; 35 µg) in acetone (0.1 ml). The mixture was applied with a brush. One week after this initial treatment the mice were tumour-promoted with the application of TPA (1.7 nmol) in acetone (0.1 ml). The mice were treated either with the test compound or with acetone (control group) before this treatment. The incidence of papillomas in the control group and test substance group were observed weekly for 20 weeks (Figure 28) (Böschen, 2008; Colombo et al., 2000; Fukuda et al., 2005; Mendoza et al., 2005; Sakurai et al., 2005; Tanaka et al., 2004).

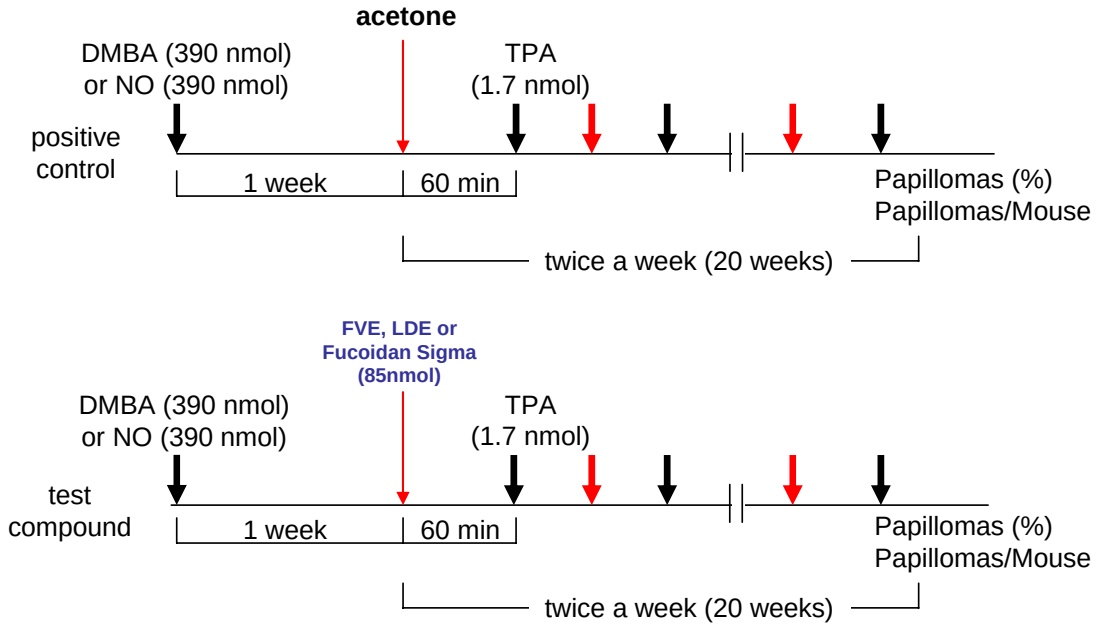



**Figure 28: *In vivo* two-stage mouse skin carcinoma test scheme modified after Böschen (Böschen, 2008)**

### 3.2.3.2 Short Term *in vitro* Bioassay for the inhibition of Epstein-Barr Virus Early Antigen (EBV-EA) activation induced by TPA

The inhibition of the TPA-induced activation of the Epstein-Barr virus (EBV) early antigen could be detected by indirect immuno fluorescence measurements. The activation of the EBV-EA was performed in Raji cells (EBV genome carrying human lymphoblastoid cells,

non producer type) by 12-O-tetradecanoylphorbol-13-acetate (TPA) as described before (Langer et al., 2006; Shirahashi et al., 1993). Therefore Raji cells ($10^6$ cells/ml) were incubated in 1 ml of a medium containing 4mM n-butyric acid as an inducer, 32 pM of TPA (20 mg/ml in DMSO), and a known amount (32, 16, 3.2, or 0.32 nmol) of the test compound at 37 °C for 48h in a $CO_2$ incubator (Langer et al., 2006). After incubation the cell suspension was centrifuged at 1000 $min^{-1}$ for 10 min and the supernatant discarded. Activated cells were stained with high titre EBV-EA-positive sera from nasopharyngeal carcinoma patients. Detection was performed with indirect immuno fluorescence technique. Viability of the cells was assayed by Trypan Blue staining (Colombo et al., 2000; Fukuda et al., 2005; Ito et al., 2005; Sakurai et al., 2005; Tanaka et al., 2004).

### 3.2.4 Antibodies against Fucoidan Sigma

This test was accomplished to generate an antibody against fucoidan. This could enhance detection of even very small amounts of fucoidan, e.g. in a blood sample. With this tool one could monitor a fucoidan therapy in a patient very well. The experiments were performed at TU Braunschweig, Institute of Biochemistry and Biotechnology, AG Dübel by Saskia Helmsing.

For the selection of the antibody libraries the immuno tubes were coated with 5 to 10 µg/ml antigen (Fucoidan Sigma) per well for the first selection round, for further panning rounds 0.5-2 µg were used. The Fucoidan Sigma was dissolved in 150 µl of 1 x PBS. Incubation took place over night at 4 °C. The antigen solution was removed and wells were filled with 2% MPBST (milk powder in PBST) for blocking at room temperature for 2h. The wells were then washed three times with an ELISA washer with PBST (PBS + 0.05% Tween 20). For a preadsorption step one well had to be prepared with 2% MPBST instead of antigen solution. Phage from the preincubation had to be filled into the antigen-coated wells and were incubated for 2h at room temperature. Unspecifically bound phage were then washed away by an ELISA washer. The specifically bound phage were eluted with 200 µl of a 10 µg/ml Trypsin solution for 30 min at 37 °C. Afterwards the titre of eluted phage was determined and the phage were used for reamplification. For titre determination of the eluted phage 50 µl of the *E.coli* XL1-Blue MRF' culture were infected with 10 µl of several dilutions from $10^{-2}$-$10^{-8}$ of the phage eluate. The mixture was incubated for 30 min at 37 °C and then plated on 2x TY-GA plates and incubated over night at 37 °C. For reamplification 50 ml of 2x TY-T medium was inoculated with *E.coli* XL1-Blue MRF' cells. Growth was monitored and at an $OD_{600}$ value of 0.5, 20 ml of the

solution were infected with 190 µl eluted phage. Infection took place for 30 min at 37 °C. Cells were collected by centrifugation (3220 g; 4000 $min^{-1}$ Centrifuge 5810 R, Eppendorf) and plated on 2x TY-GA agar plates. The bacteria were scraped with 5 ml 2x TY-GA the next day and collected in a Falcon tube. 50 ml of the 2x TY-GA medium was inoculated with 50 to 200 µl of the bacteria ($OD_{600}$ <0.1) and incubated until OD reaches 0.4-0.5.

Around 2.5 x $10^9$ bacteria were then infected with M13K07 phage (helper phage) with a MOI of 1:20 (5 x $10^{10}$ pfu) and incubated for 30 min at 37 °C without agitation and 30 min with an agitation rate of 250 $min^{-1}$. The rest of the bacteria were stocked in 20% glycine and stored at -80 °C.

For the production of single clones, a 96 well micro titre plate was filled with 150 µl 2x TY-GA medium each well. 92 antibody clones were picked from the titre plate of the last panning round with a sterile pipette tip and were used to inoculate the medium. Four wells (H3, H6, H9 and H12) are not inoculated by clones but were used as positive and negative controls. H9 and H12 (positive controls) were inoculated with XL1-Blue:pHal14-D1.3 and the plate was incubated at 37 °C at 1200 $min^{-1}$ (→ master plate).

A new 96 well micro titre plate was filled with 180 µl 2x TY-GA medium each well and 10 µl of the overnight cultures was added. The master plate could be stored at 4 °C for up to two weeks or can be glycine stocked (20% glycine) at -70 °C. Incubation of the new cultures were performed at 37 °C for 2h at 1200 $min^{-1}$. The bacteria were harvested with centrifugation (4000 $min^{-1}$; 3220 g; Centrifuge 5810 R, Eppendorf, rotor A-4-81) for 10 min. The supernatant was discarded by carefully pipetting at the outside margin in order not to destroy the pellet. The pellet was dissolved in 180 µl 2x TY-A medium with 50 µM IPTG and incubated over night at 30 °C and 1200 $min^{-1}$. Bacteria were centrifuged at 3220 g (4000 $min^{-1}$). 180 µl of the supernatant was taken to a new micro titre plate (contains the soluble antibody). These soluble antibodies could then be used in ELISA analysis.

For ELISA analysis Fucoidan Sigma (100 ng to 1 µg) was dissolved in coating buffer (PBS) and filled in the micro titre plate. The mixture was incubated over night at room temperature. The wells were washed three times with PBST and were then filled completely with blocking solution (2% MPBST). The blocking mixture was incubated for 1.5h at room temperature. The wells were then washed three times with an ELISA washer with PBST. The coated wells were then filled with 50 µl blocking solution and 50 µl soluble antibody (from the production plate) and the mixture was incubated for 1.5h at room temperature. The wells were then washed with PBST in an ELISA washer and 100 µl

Mouse α-myc-tag (1:$10^x$ in blocking solution; $^x$ depending on the production) was added and incubated for 1.5h at room temperature. After washing three times with PBST in the ELISA washer 100 µl Goat α-Mouse IgG (Fab specific) HRP (1: 10,000 in blocking solution) was added and incubated for 1.5h at room temperature. After three washing steps with PBST in the ELISA washer 100 µl substrate solution (TMB substrate for the HRP) was added and incubated until the blue colour was sufficient for measurement in the ELISA reader (agitation at 750 $min^{-1}$ once in a while). Measurement was carried out at 655 nm. Stopping of the reaction is done by 100 µl of 1 N $H_2SO_4$. Measurement of the yellow colour was done at 450 nm in the ELISA reader (reference wave length of 620 nm).

## 3.3 Microorganisms with a Fucoidan-Degrading Potential

### 3.3.1 Isolation Methods

Some of the strains used in this project were isolated at Wilhelmshaven, Germany, in 2005 and 2007. To cultivate both bacteria and fungi from algal pieces different *Fucus vesiculosus* thalli were collected, washed with North Sea water and kept separately, cold and dark until further preparation. For the isolation the thalli were sterilised on their surface with sodium dodecylsulfate (0.1%) for 1 min, washed with sterile distilled water for 1 min and then sterilised with sodium hypochlorite (3%) for 10 to 15 sec. The thalli were then washed three times with sterile distilled water for 3 min each time (Zuccaro et al., 2003).

It was very important that the isolation procedure was performed contemporary to collection in order to suppress mouldering of the fungi. After sterilisation the thalli were parted with a sterile knife into three sections: the upper part (head), the middle part (body), and the lower part (foot), as they were assumed to contain different amounts of polysaccharides. In the head there are smaller and bigger bubbles that are filled with air to keep the algae upright under water. In these bubbles there is a polysaccharide slime that might be very interesting for the detection of marine organisms. The body only contains small amounts of polysaccharides. Microorganisms can be found in this part if the thalli were harmed before collection. The foot contains quite high amounts of polysaccharides that are responsible to fix the algae to stones and other permanent undergrounds. There, bacteria and fungi should be found, that are able to degrade the targeted polysaccharides. In Figure 29 a thallus is shown.

**Figure 29: *Fucus vesiculosus* thallus (http://upload.wikimedia.org/wikipedia/commons/9/9e/Fucus_vesiculosus_Wales.jpg)**

The algal parts were then plated onto different agar plates with different ingredients, the exact recipes of which are listed in the appendix (Chapter 9.11; Table 60-64). In order to distinguish between bacteria and fungi some agar plates contained different added antibiotics such as penicillin (60 mg/l), streptomycin (80 mg/l) and tetracycline (50 mg/l). The agar plates were incubated at room temperature for more than 5 weeks. Every day the plates were checked for new microorganisms. The time of development, the algal part, and the contents of the agar plate were noted and the new microorganism was isolated onto a new agar plate of the same kind. Using this method, more than 80 fungi (isolated 2005) could be found. The different bacteria (isolated 2007) could not be distinguished from each other yet and are still connected in communities.

### 3.3.2 Screening Methods

Our institute has a large number of marine bacteria and marine fungi that were isolated during this and other projects. It is highly probable that one of these strains also is a potent degrader of fucoidans from brown algae. Unfortunately, not all of these strains could be tested in this project. Screening was performed as described in chapter 3.1.12.3 by either cultivating the microorganism on the polysaccharide or by testing the supernatants and cell lysates on the polysaccharides. Any observed activity was considered indicative of an enzyme producer. These fungi and bacteria were then analysed further. Fungi and bacteria that are well known as ubiquitous microorganisms were ignored in favour of marine fungi and bacteria.

### 3.3.3 Cultivation Methods and Media

Two different cultivation methods were applied during the project: Solid state cultivation and liquid cultivation.

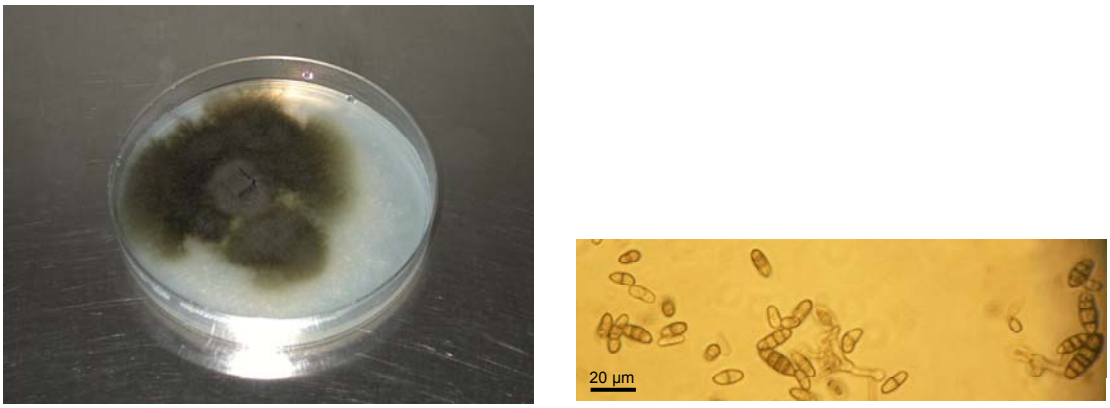
Solid state cultivation included the cultivation on solid media like starch, straw or wheat bran. Liquid cultivation was performed with different media in different amounts starting at 100 ml shake flask up to 8 l bioreactor volume. As every strain required special cultivation conditions the cultivation procedure is given in each strain's section or in the appendix (chapter 9.11). One important medium that was used to analyse the ability of the microorganisms to utilise different C-sources is a general salt medium, called 'minimal medium' further on. The constituents of this medium are stated in Table 11.

**Table 11: Minimal medium for C-source consumption experiments**

| Compound | Amount |
|---|---|
| NaCl | 28.13 g |
| KCl | 0.77 g |
| $CaCl_2$ x 2 $H_2O$ | 1.60 g |
| $MgCl_2$ x 6 $H_2O$ | 4.80 g |
| $NaHCO_3$ | 0.11 g |
| $MgSO_4$ x 7 $H_2O$ | 3.50 g |
| Distilled water | 1000 ml |

### 3.3.4 *Dendryphiella arenaria* TM 94

*Dendryphiella arenaria* TM 94 was isolated in 1998 by the Fachhochschule Emden (Wu et al., 2002) from sand of the beach in Copenhagen. It is a marine fungus with high spore production. *Dendryphiella arenaria* shows very high similarities to *Dendryphiella salina* (Abbot et al., 1974); so throughout the project the strain was named back and forth due to uncertainties in this matter. Pugh (Pugh and Nicot, 1964) describes the problem to distinguish *Dendryphiella salina* from *Dendryphiella arenaria* (Nicot, 1958) since the strains have so many common features. The conidia of *Dendryphiella arenaria* TM 94 are variable septated and have a quite smooth surface in comparison to *Dendryphiella salina* whose surface is somewhat roughened. This observation and the fact that *Dendryphiella salina* possesses mostly three septated conidiophores lead to the conclusion that the isolate is *Dendryphiella arenaria*. Cultivation was performed as solid state cultivation on straw and wheat bran as well as in liquid culture. The recipes are listed in the appendix. When liquid cultivation was applied a common fungus medium was (fungus medium 1; see chapter 9.11) used. Figure 30 shows a the fungal mycelium of *Dendryphiella arenaria* TM 94 on an agar plate as well as spores under the microscope.




A B

**Figure 30: *Dendryphiella arenaria* TM 94 mycelium cultivated on potato-dextrose-agar; B = *Dendryphiella arenaria* TM 94 spores cultivated on potato-dextrose-agar**

### 3.3.5 Self Isolate WHV059

Self isolate WHV059 is a marine fungi that was isolated out of the foot of the alga *Fucus vesiculosus* in April 2005 at Wilhelmshaven Südstrand (N 53°51', E 8°14'). Initial analyses come to the conclusion that it is *Acremonium spec*. (Alga Zuccaro, personal communication) even though it is a different strain than the one described in Zuccaro (Zuccaro et al., 2004). The mycelium is slightly rosé or orange. There is no high spore production and the fungus grows very slowly. Cultivation of this fungus is performed on potato-carrot-agar as well as in potato-carrot liquid medium at 27 °C (Zuccaro et al., 2004). Figure 31 shows a picture of Self Isolate WHV059.

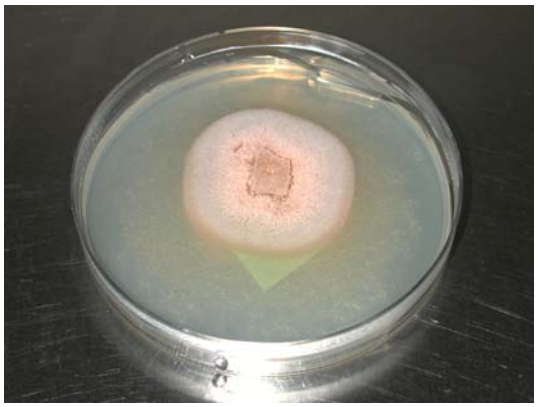
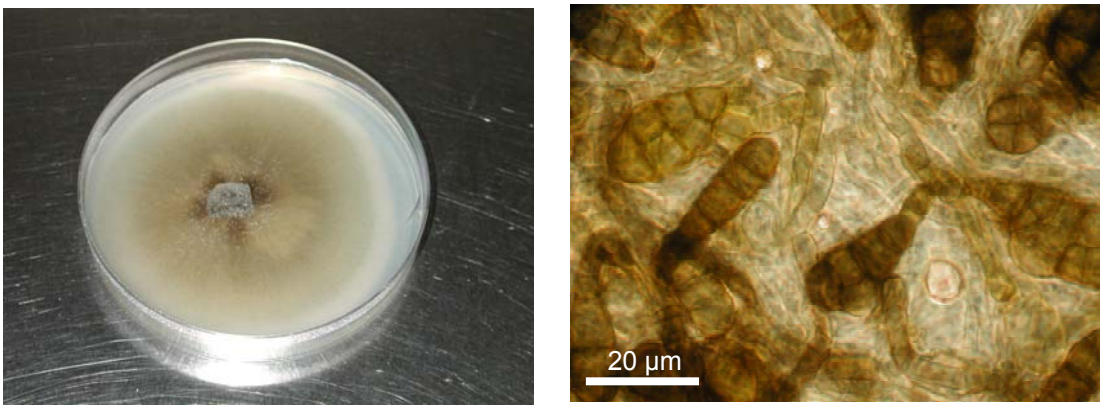


**Figure 31: Self Isolate WHV 059 mycelium cultivated on potato-dextrose-agar**

### 3.3.6 Self Isolate WHV012

Self isolate WHV012 is also a marine fungi and that was isolated out of the foot of the alga *Fucus vesiculosus* in April 2005 at Wilhelmshaven Südstrand (N 53°51', E 8°14'). The fungus is slightly faster growing than the isolate WHV059. First analyses indicate that the fungus appears to be of the genus *Alternaria* (Siegfried Draeger, personal communication). The spore production is quite slow (2 to 3 weeks). Cultivation was done on potato-carrot-agar at 27 °C as well as in liquid medium. Figure 32 shows the mycelium of Self Isolate WHV012 as well as spores under the microscope.




A B

**Figure 32: A = Self Isolate WHV012 mycelium cultivated on potato-dextrose-agar; B = Spores of Self Isolate WHV012 cultivated on potato-dextrose-agar.**

### 3.3.7 *Saccharophagus degradans* DSM 17024

*Saccharophagus degradans* DSM 17024 was chosen because it was stated in literature (Ekborg et al., 2005; Gonzalez and Weiner, 2000) that this micro organism is able to degrade fucoidan – although the source of fucoidan was not specified. It is also a very interesting bacterium because it is able to cleave at least 10 different marine poly-saccharides. It could represent a positive control for many cleaving patterns of marine polysaccharides that could occur. *Saccharophagus degradans* DSM 17024 was isolated from the surface of degrading salt marsh cord grass (Andrykovitch and Marx, 1988) and is described as gram-negative, motile, heterotrophic, pleomorphic, rod-shaped, aerobic, catalase-positive, and oxidase-positive (Ekborg et al., 2005). It requires sea salts for growth (half strength marine agar from Difco (2216)) and reaches a size of 1.5-3 µm in length and 0.5 µm in width. The colonies are cream in colour and turn black upon eumelanin production. *Saccharophagus degradans* DSM 17024 pits agar plates as it is able to degrade agarose as well (see Figure 33). The optimal cultivation temperature lies between 4 and

37 °C. In our experiments *Saccharophagus degradans* DSM 17024 was cultivated mostly at 27 °C. pH optimum for growth is 7.5 with a range of 4.5 to 10. Figure 33 shows a picture of *Saccharophagus degradans* DSM 17024 colonies on Difco Medium 514a. The pits in the agar are clearly visible.

**Figure 33: *Saccharophagus degradans* DSM 17024 colonies cultivated on half strength Difco medium 514. Great pits were visible on the agar plate.**

### 3.3.8 *Pseudoalteromonas atlantica* DSM 6839

*Pseudoalteromonas atlantica* DSM 6839 was chosen because of its ability to degrade "fucoidin" (Yaphe and Morgan, 1959). The fucoidin was isolated from *Fucus vesiculosus* and can be equated with the fucoidan known today. The utilisation of fucoidan was determined from the fucose equivalents of aliquots removed from the control and fermentation flasks (Yaphe and Morgan, 1959). *Pseudoalteromonas atlantica* DSM 6839 was able to degrade fucoidan and had a pH optimum of 7 for this enzymatic activity. Another polysaccharide degrading enzyme detected in *Pseudoalteromonas atlantica* DSM 6839 was an agarase (Perepolov et al., 2005). *Pseudoalteromonas atlantica* DSM 6839 produces an exopolysaccharide to adhere to surfaces (Bartlett et al., 1988). This adhesion makes it difficult to grow in liquid cultures.

### 3.3.9 *Pseudoalteromonas carrageenovora* DSM 6820

*Pseudoalteromonas carrageenovora* DSM 6820 was also chosen because of its ability to degrade "fucoidin" (Yaphe and Morgan, 1959). The utilisation of fucoidan was determined from the fucose equivalents of aliquots removed from the control and fermentation flasks (Yaphe and Morgan, 1959). Several carragenases were isolated from the bacterium (Knutsen and Grasdalen, 1992; Ohta and Hatada, 2006). Difficulties in handling *Pseudoalteromonas carrageenovora* DSM 6820 could arise because it is forming a biofilm on marine surfaces, making biomass analysis very demanding.

### 3.3.10 *Pedobacter heparinus* DSM 2366

*Pedobacter heparinus* DSM 2366 was chosen because of its ability to degrade heparin. It is described in literature (Steyn et al., 1998) that *Pedobacter heparinus* DSM 2366 is a heparinase-producing, obligatory aerobic gram-negative rod varying in form. The length varies between 0.7 to 6 µm and 0.5 µm in width with rounded or slightly tapering ends. The colour of the colonies ranges from creamy to dirty yellow depending on the agar used. In our experiments *Pedobacter heparinus* DSM 2366 was grown on DSMZ medium 1, like all soil bacteria. *Pedobacter heparinus* DSM 2366 possesses three different heparinases of different size and substrate specificity (Shaya et al., 2006). As there is a similarity between the fucoidanolytic system and the heparinatic system (Kitamura et al., 1992; Kusaykin et al., 2003) *Pedobacter heparinus* DSM 2366 was used to calibrate the newly developed analytic systems such as SE-HPLC with the oligosaccharides produced by the strain. Another idea was to gain information about the FVEs, LDE and Fucoidan Sigma and their potential structure similarities to heparin by degrading them with *Pedobacter heparinus* DSM 2366. For example, heparinase 1 from *Pedobacter heparinus* DSM 2366 (EC 4.2.2.7) performs an eliminative cleavage of polysaccharides containing (1->4)-linked D-glucuronate or L-iduronate residues and (1->4)-α-linked 2-sulfoamino-2-deoxy-6-sulfo-D-glucose residues to give oligosaccharides with terminal 4-deoxy- α -D-gluc-4-enuronosyl groups at their non-reducing ends.

### 3.3.11 Tests with Commercially Available Enzymes

For the enzyme tests, commercially available enzymes were tested with their optimal substrates as well as with FVEhigh, FVElow, Fucoidan Sigma, LDEhigh and p-nitro phenyl-sulphate. Analysis was performed in 96 well plates in a Multiskan Ex (Thermo Labsystems) at 405 nm for the optimal substrates and after an additional sugar detection

test (colorimetric tests) in a spectrophotometer (Pharmacia Biotech; Ultrospec 3000) for the samples to be analysed.

The commercial enzymes used in this project were

1. Laminarinase (Endo-β-1,3(4)-Glucanase) from *Trichoderma sp.*)
2. Fucosidase (α-1→(3,4)-Fucosidase) solution from *Xanthomonas manihotis*)
3. Fucosidase (α –L- Fucosidase) solution from bovine kidney
4. Glucosidase (α –D- Glucosidase) from *Bacillus stearothermophilus*

**Laminarinase**

For the laminarinase, FVEhigh, Fucoidan Sigma, and laminarin was applied. Substrate solutions were dissolved in 50 mM sodium-acetate buffer (pH 5) in a concentration of 10 mg/ml. Enzyme solution was also dissolved in 50 mM sodium-acetate buffer. A concentration of 0.01 U/2.4 ml was applied in the tests, resulting in a concentration of 0.00417 U/ml. 100 µl enzyme solution was added to 100 ml substrate solution and incubated at 37 °C in a water bath. After different periods, samples were taken and the enzyme reaction was stopped by boiling in a 100 °C water bath. The samples were frozen at -20 °C until further analysis with colorimetric tests.

**α-1→(3,4)-Fucosidase (from *Xanthomonas manihotis*)**

α-1→(3,4)-Fucosidase was tested with 4-nitrophenyl- α-L-fucopyranoside as well as with the other substrates. The substrates were dissolved at a concentration of 10 mg/ml in 250 mM sodium-phosphate buffer (pH 5) to generate a concentration of 0.00208 U/ml. 100 µl enzyme solution were added to 100 µl of substrate solution and incubated at 37 °C in a water bath. After different periods, samples were taken and the enzyme reaction was stopped by boiling in a 100 °C water bath. The samples were frozen at -20 °C until further analysis in a spectrophotometer.

**α-L-Fucosidase (from bovine kidney)**

α-L-Fucosidase was tested with 4-nitrophenyl- α-L-fucopyranoside as well as with the other substrates. The substrates were dissolved at a concentration of 1 mg/ml in enzyme buffer (3.2 M $(NH_4)_2SO_4$, 10 mM $NaH_2PO_4$, 10 mM citrate; pH 6.0). 4 µl enzyme solution were added to 46 µl substrate solution. After different time intervals the reaction was stopped with 250 µl of $Na_2CO_3$-solution (1 M).

**Glucosidase from *Bacillus stearothermophilus***

α-D-Glucosidase from *Bacillus stearothermophilus* is also known as α-D-glucoside glucohydrolase or maltase. α-Glucosidase is active on p-nitrophenyl- α-D-glucoside. In this thesis it was applied on p-nitrophenyl- α-L-fucopyranoside and p-nitrophenyl-sulphate to act as a negative control. The substrates were dissolved at a concentration of 10 mg/ml in 250 mM sodium-phosphate buffer (pH 7) to generate a concentration of 0.00208 U/ml. 100 µl enzyme solution were added to 100 µl of substrate solution and incubated at 37 °C in a water bath. After different periods, samples were taken and the enzyme reaction was stopped by boiling in a 100 °C water bath. The samples were frozen at -20 °C until further analysis in a spectrophotometer.

## 3.4 Protein Analytics

### 3.4.1 Protein Determination by Bradford

The amount of protein in a supernatant or other enzyme preparation was determined using the Bradford method. First, either the commercially available Bradford solution or a self made solution (recipe see appendix) was diluted 1:4 in water. Different concentrations of a BSA (Bovine Serum Albumin) solution were prepared as standards. For this application, concentrations of 0.2, 0.4, 0.6, 0.8, and 1 g/l in phosphate buffer of the desired pH was used. The protein concentration of the enzyme preparation should lie within this range. For analysis, 25 µl of the sample (BSA or enzyme preparation) was mixed with 1250 µl of the diluted Bradford solution. The mixtures were incubated for at least 5 min but no longer than 30 min. The samples were then analysed by a spectrophotometer at 595 nm in plastic cuvettes.

### 3.4.2 Protein Determination by SDS-PAGE

The protein determination with SDS-PAGE should give the opportunity to see the different proteins produced while estimating their size. These methods will be called “protein pattern determination” further on.

As the amount of protein produced in the different cultivations was not very high, the protein had to be concentrated before analyses. Two possibilities were tested in the project:

1. Ultrafiltration
2. Acetone Precipitation

For ultrafiltration special filtration caps were used (Vivaspin 6 ml, Sartorius Göttingen) with a molecular weight cut off (MWCO) of 3000 Da. This makes it possible to get rid of inorganic salts polluting the media and to obtain just proteins. The filtration caps were filled with 400 µl of the sample and centrifuged for 1h at 4000g (Centrifuge 5810 R, Eppendorf). The concentrate could then be taken and analysed in the SDS-PAGE (recipe for the gels see appendix chapter 9.4).

The second possibility was acetone precipitation. In this method 200 µl of the sample were mixed with five volumes of ice-cold acetone and kept in the freezer for 2h. Afterwards the precipitated proteins were resuspended in 40 µl Sörensen-phosphate buffer (pH 7) and were dried at room temperature. The concentrate could then be used in the SDS-PAGE.

For the SDS-PAGE 40 µl of the concentrated sample were mixed with 10 µl of Laemmli-buffer (5x) and cooked for 10 min. 25 µl of this mixture was then applied on the SDS gel (12% running gel, 4% collecting gel). As protein standard a prestained PAGE ruler (Fermentas) was used. Only 2 µl of this marker were applied on the gel. The running buffer consisted of a usual running buffer (see appendix) with SDS (pH 8.3) at 10 mA per gel. Visualisation of the proteins was done by silver nitrate staining (see appendix chapter 9.6).

## 3.5 Extraction of Fungal Metabolites

Literature studies (de la Cruz et al., 2006) revealed that *Dendryphiella* species inhibit the growth of several microorganisms including *Microbotryum violaceum* (formerly *Ustilago violacea*). *Dendryphiella arenaria* TM 94 was plated on malt extract peptone yeast extract agar (Schulz et al., 1995) and incubated in darkness for 4 weeks at 25 °C. Thereafter the production of metabolites of *Dendryphiella arenaria* TM 94 and self isolate WHV059 was analysed. Unlike the experiments of Schulz, *Dendryphiella arenaria* TM 94 and self isolate WHV059 were cultivated in liquid culture (MPY; malt extract peptone yeast extract without agar). 250 ml of the medium was incubated in 1000 ml Erlenmeyer flasks for 4 to 7 days at 27 °C at 100 $min^{-1}$. Liquid cultivation was inoculated with spore solution of the two fungi. Sole medium acted as a reference. After incubation the cultivation broth was centrifuged at 3000 $min^{-1}$ (Centrifuge 5810 R, Eppendorf) for 30 min. The supernatant was collected and stored at 4 °C until further treatment. The pellet was lyophilised and resuspended in chloroform:methanol (1:1). The reference medium as well as the supernatant were also lyophilised and resuspended in tert.-butylmethylether for extraction.

Extraction takes place through liquid dispersion in a separating funnel. Organic phases were collected, dried with sodium sulphate and filtrated through a normal fluted filter. In some cases the cell extract contained rests of cell debris. These debris were removed with membrane filtration (RC-L 55; 45 µm Schleicher and Schuell). The filtrates were then parted and filled into two round bottom flasks as one part was used for TLC analysis and the other part was used for antibacterial measurements. After vaporisation of the extraction medium the amount of extracted compounds could be determined (dead weight if the round bottom flask is distracted). Resuspension of the extracts was carried out according to the amount of extracted compounds in order to get the same concentrations. The extracts used for bioactivity analysis were resuspended in DMSO (dimethylsulfoxide). The contents of the second round bottom flasks were either suspended in MTBE (methyl-*tert*. butyl ether; media and supernatants), or in chloroform:methanol (1:1) (cell extracts). The amount of solvent used for the resuspsension had to be chosen so that the concentration of the resuspended extracts was 100 mg/ml. They were stored in Pyrex tubes at 4 °C until further usage.

### 3.5.1 TLC Analysis of the Extracts

10 µl of the resuspended extracts (resuspension in MTBE or chloroform:methanol (1:1)) were applied on a silicagel 60 $F_{254}$ aluminium TLC plate (Merck). Development of the silica plate took place in a TLC chamber with 4% methanol in chloroform. The dried TLC plates were visualised with different reagents according to the compound to be detected. Anisealdehyde visualises polyalcohol, steroids and terpenes. 5% sulfuric acid in methanol visualises organic substances. After spraying with the visualisation agents the TLC plate was developed 5 to 10 min at 110 °C.

### 3.5.2 Tests for Anti-microbial Activity

The cell extracts and the extracts of the supernatants that are resuspended in DMSO are used for the determination of activities against several testorganisms. Depending on the testorganism, different agar plates with either YPD, FP, or LB medium are marked with a cross on the backside into four zones. The scheme can also be seen in Figure 34. In every quarter a sterile test paper (6 mm round filter) is placed. These paper snippet is soaked with 10 µl extract ( 50 mg/ml in DMSO) with a Hamilton syringe.

A spore suspension of the test organism is then produced. Thereafter 3 to 4 injection loops of the organism or 2 $cm^2$ of the agar plate (fungi) are dispersed in 100 ml sterile dest.$H_2O$

(for *Microbotryum violaceum*) or 100 ml 0.9% NaCl in dest $H_2O$ and kept for diffusion in the hood for 2h. After diffusion time the agar plates with the paper snippets are sprayed with the test organism solutions. Pure DMSO is used as a negative control. Positive controls are agar plates containing amphotericin B, penicillin G, or chloramphenicol (depending on the test organism). Incubation takes place for three days at room temperature (22 °C).

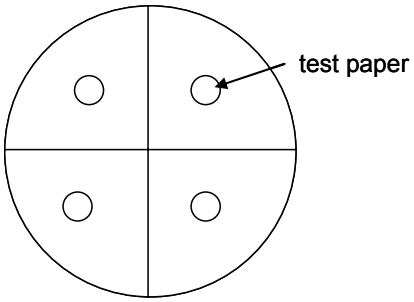



**Figure 34: Labelling of the agar plates for antibacterial tests**

# 4 Results

In the following paragraphs selected results are presented. They are divided into three major parts. The first part deals with the sulphated polysaccharides, their extraction, characterisation, and modification. The second part examines the bioactivity of these polysaccharides and their potential applications in medicine. The third and last part deals with the microorganisms possessing a potential fucoidan-degrading ability as well as with commercial enzymes with a potential to degrade fucoidan. In addition to these major parts, the extraction of fungal metabolites is described.

## 4.1 Sulphated Polysaccharides/Oligosaccharides

### 4.1.1 Extraction Procedure

The extraction procedure was adapted to Black (Black, 1954; Black et al., 1952) and optimised with regards to the yield of fucoidan. Several tests were performed to analyse the quality and amount of the produced heterogeneous polysaccharides, leading to the extraction procedure stated in chapter 3.1.1. Figure 35 shows the established reaction scheme with regards to the yield.

### 4.1.2 Monosaccharide Composition

In order to elucidate the monosaccharide composition of the four different extracts (FVEhigh, FVElow, Fucoidan Sigma, LDEhigh) the polysaccharide structures were degraded by methanolisation with 0.625 N HCl and the fractions were analysed by Dr. Manfred Nimtz at the HZI, Braunschweig. Figure 36 shows exemplarily the GC results for FVEhigh. The upper diagram shows the GC measurement of all molecular weights and the lower graph shows the GC measurement of the mass fragments 204 and 217 which were known to represent the sugar components. By comparison of the two diagrams one can see, that FVElow is consisting mainly of sugars and shows only minimal pollution with other compounds. The main component of FVEhigh is fucose. The system was calibrated with known amounts of the potential mono sugars present in fucoidan. Figure 37 shows the calculated amounts of different monosaccharides in the analysed polysaccharides.

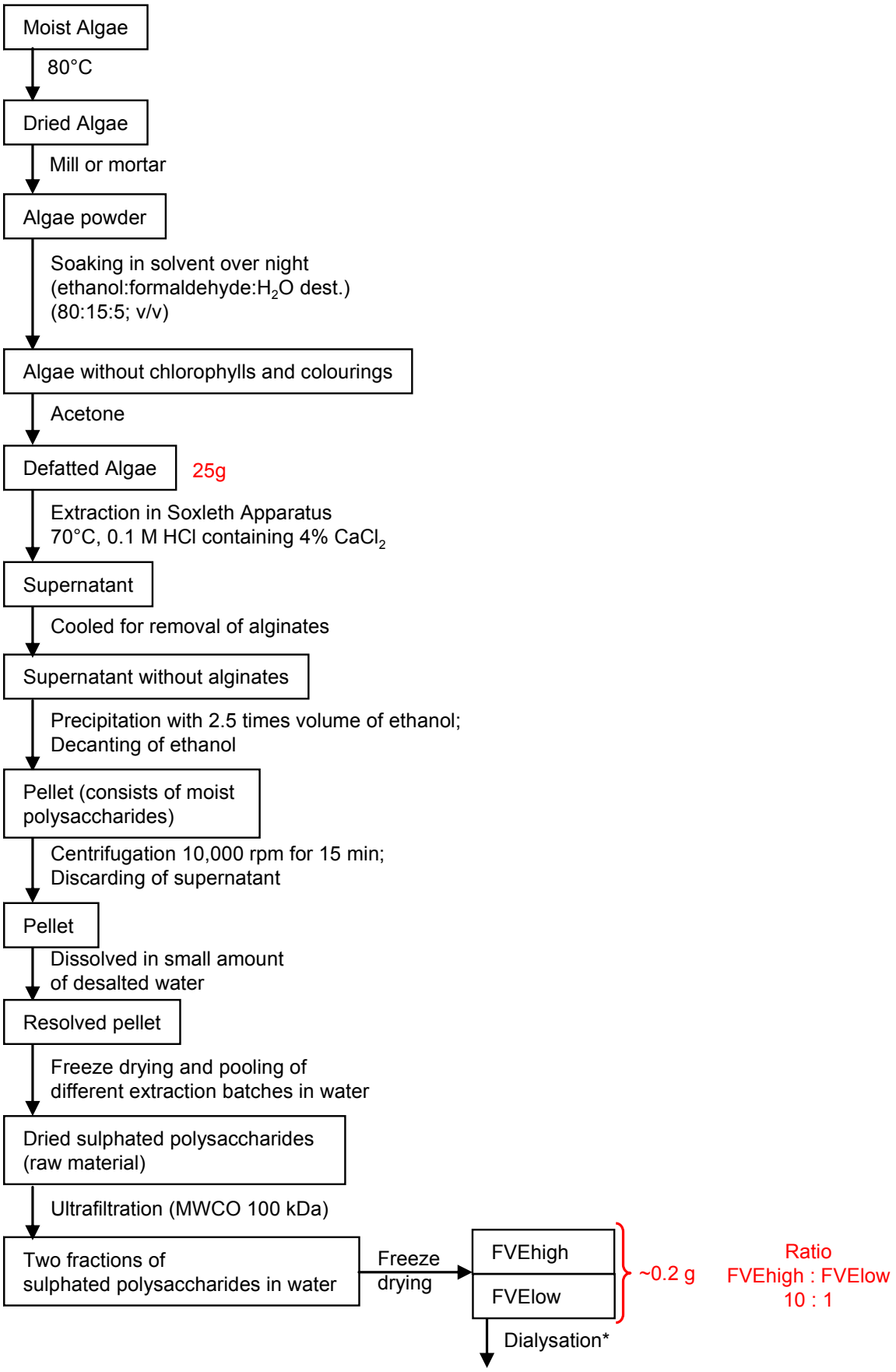



**Figure 35: Extraction scheme for fucoidan from brown algae: *Fucus vesiculosus* and *Laminaria digitata* (*Laminaria digitata* was purchased as powder); *FVElow and Fucoidan Sigma needed dialysation.**

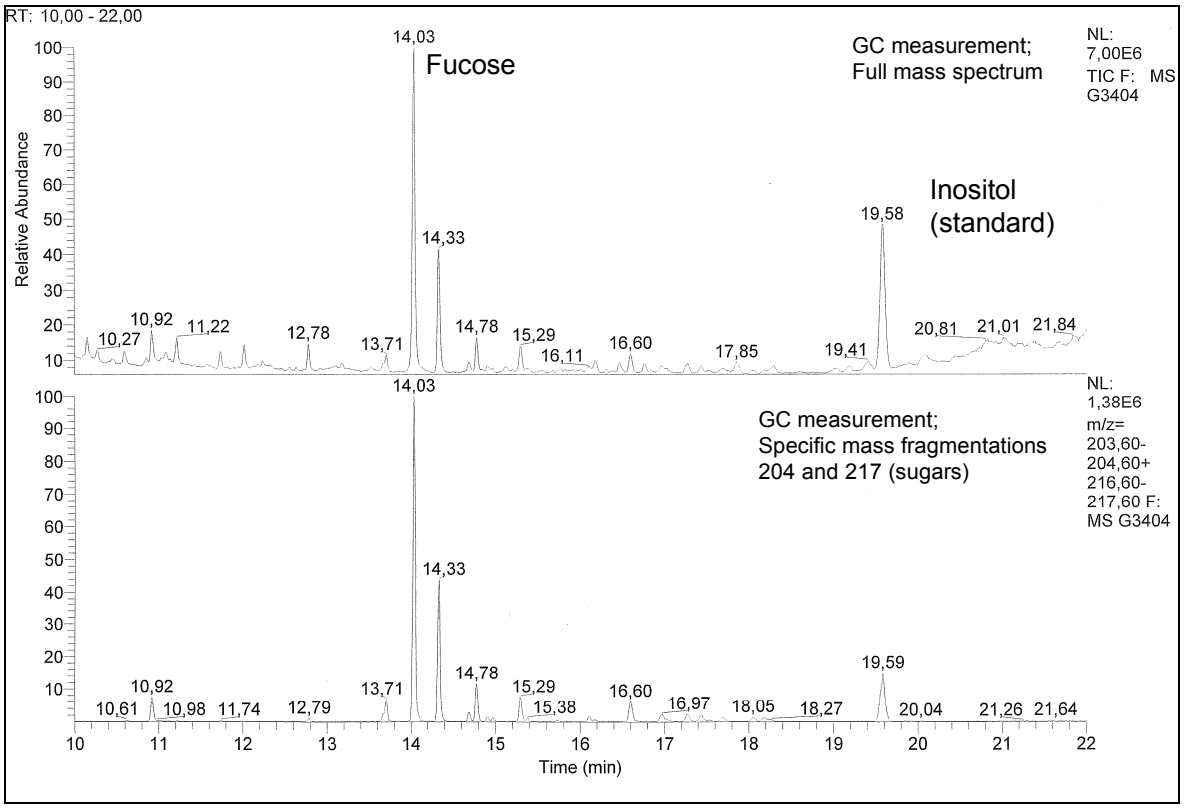



**Figure 36: GC diagrams of the GC/MS result for FVEhigh; RT = retention time; NL = intensity of base peak; TIC = total ion current; m/z = intensity distribution of the m/z range; Xcalibur™**

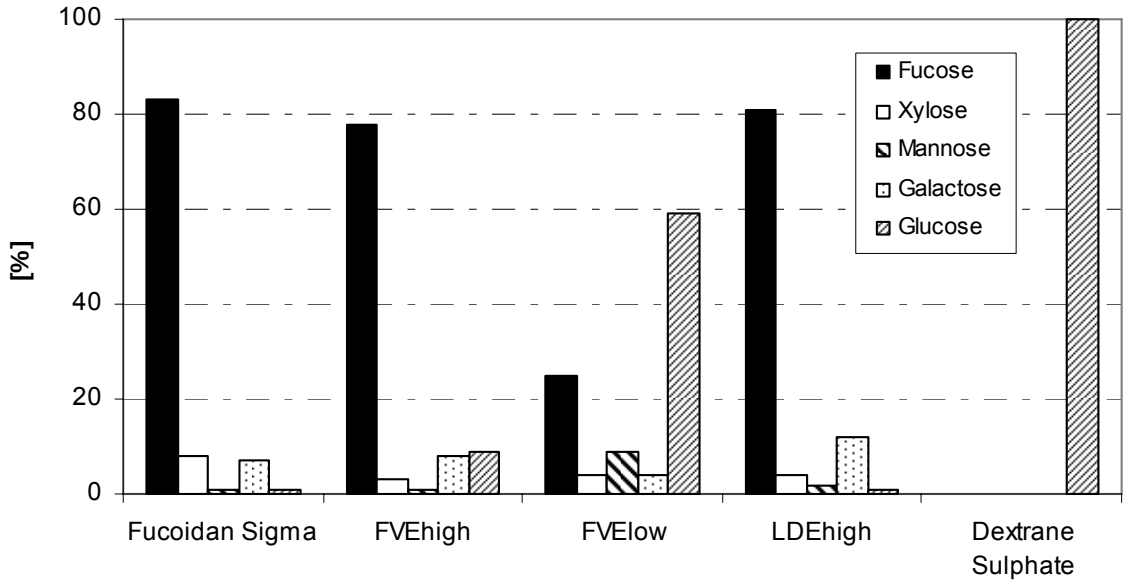



**Figure 37: Comparison of the monosaccharide composition of different heterogeneous polysaccharides**

All measurements showed that fucose is the main component of all three high molecular weight extracts. FVElow mainly contained glucose. Dextran sulphate consists of 100%

glucose as expected and could thus act as a negative control. It is interesting to see that the bigger fucoidans contain almost the same amounts of fucose and mannose. The amount of xylose is slightly higher in the Fucoidan Sigma and the amount of galactose is increased in the LDEhigh. The mannose content of FVElow exceeds the content of the bigger polysaccharides. The xylose content is slightly higher in Fucoidan Sigma. The content of glucose in FVEhigh is higher than in Fucoidan Sigma and LDEhigh.

### 4.1.3 Elemental Analysis

Elemental analysis was done in the laboratory of Ilse Beetz, Kronach, Germany. The analysis was done with the FVEs high and low (dialysed; Visking® dialysis tubing, regenerated cellulose; 12-14 kDa, pore diameter 25 Å) as well as for Fucoidan Sigma (as purchased, without further treatment). Three different analyses for the different constituents were necessary. For evaluation the two main constituents fucose and sulphated fucose were calculated stoichiometrically as presented in Figure 38.

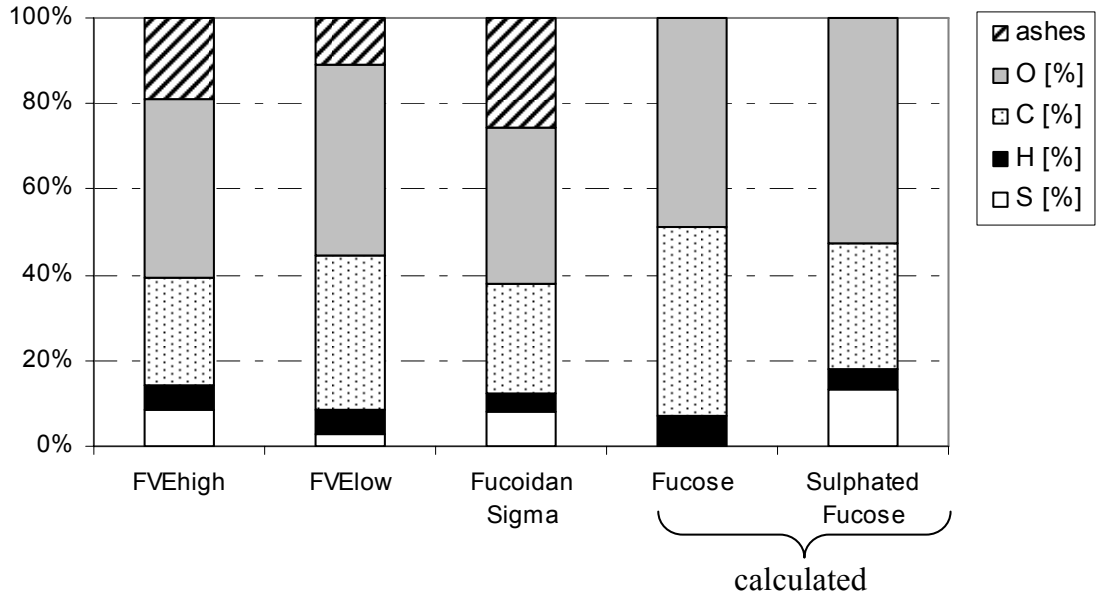



**Figure 38: Elemental analysis performed in the laboratory of Ilse Beetz, Kronach, Germany.**

Based on the elemental analyses, it appears that approximately every second fucose molecule in the polysaccharides is sulphated. If there are many other monosugars present the sulphation degree per molecule is even higher. It is very interesting that the amount of sulphur measured in FVEhigh and Fucoidan Sigma is almost the same whereas the sulphation degree of FVElow is relatively minor.

### 4.1.4 Molecular Structure Proposal

Taking the performed analyses into account, a structure for the FVEhigh with 78% fucose can be assumed, of which every second fucose is sulphated. Other important monosaccharides are galactose and glucose, with around 10% of the total content. Minor constituents are xylose and mannose. As the sulphation positions and the binding between the different sugars are unknown, the structure proposed in Figure 39 is considered to be very uncertain. Only α-bonds are assumed in this proposal since these bonds are the most common in marine molecules.

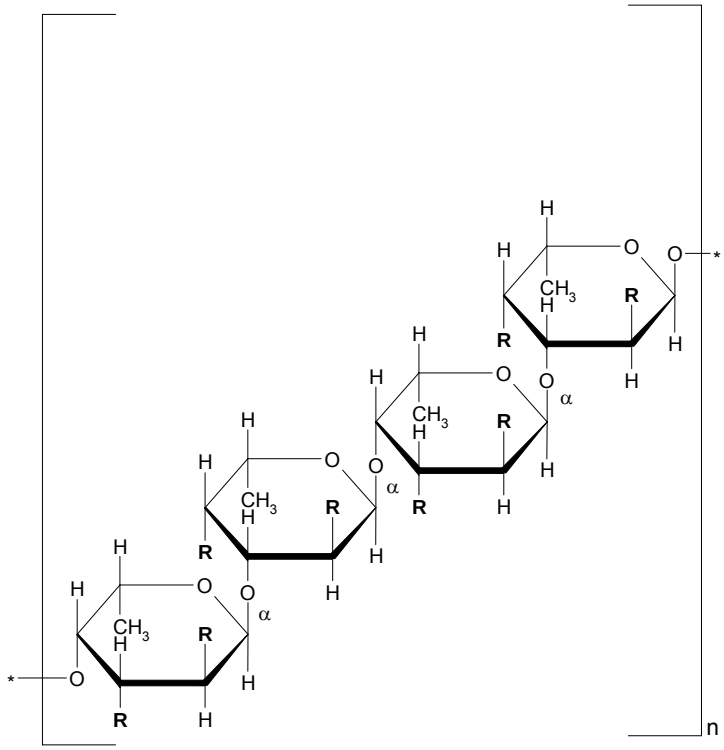



**Figure 39: Structural proposal for FVEhigh; R = $OSO_3^-$, OH groups, or other monosaccharides such as glucose, galactose, xylose or mannose**

### 4.1.5 SE-HPLC Method Development

For the detection with a refractive index detector the optimal solvent was determined and the optimal flow parameters were elucidated. As optimal solvent a 0.01 M $LiNO_3$ solution in $H_2O$ was found as the baseline and the signal peaks could be distinguished very well with this combination. This solvent was suggested in literature by Karlsson (Karlsson and Singh, 1999). The flow of 1 ml/min allowed the peaks to be distinguished very well from each other. A faster flow was not possible due excessive backpressure in the column. Tests

with a slower flow led to poor resolution between the different peaks (data not shown). Running time was estimated to 40 min.

### 4.1.6 C-PAGE Development

In order to get a suitable method to detect bigger and smaller fucoidans, the C-PAGE method was adapted from Descamps (Descamps et al., 2006). The system described was used in standard mini gels and therefore lacks the possibility to separate molecules of a high variety in molecular weight. For this project a DNA Sequencer system from OWL was used as gel stand and the spacer were custom-made to 1 mm in thickness and 450 mm in length. The resolution of FVEs in this gel was much better than in the standard mini gels. The staining procedure was successfully adapted. The finally established method is shown in chapter 3.1.4.

### 4.1.7 Size Determination

After setting up the SE-HPLC method, a first calibration with different polysaccharides like heparin, laminarin, dextrans, dextran sulphates and Fucoidan Sigma was performed. General size determination was done using a RI detector. Figure 40 shows a calibration performed with dextrans of different molecular weights dissolved in 0.01M $LiNO_3$. It can be seen that the resolution of these linear molecules was performed very well.

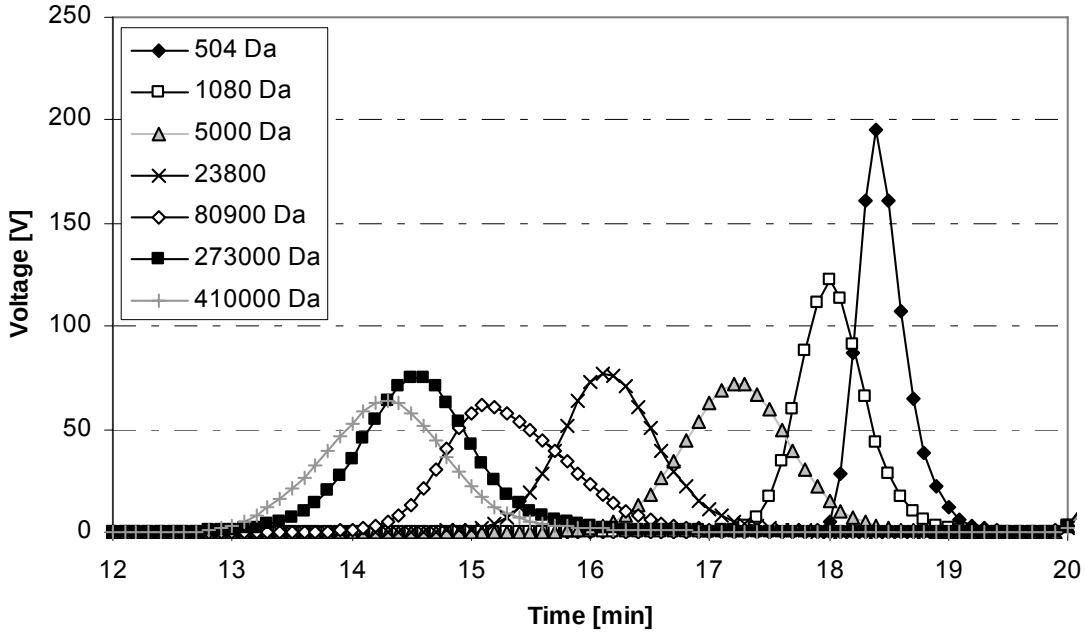



**Figure 40: Different molecular weight dextrans dissolved in 0.01M $LiNO_3$, flow rate 1ml/min, concentration 2g/l**

Figure 41 shows different molecular weight dextran sulphates dissolved in 0.01 M $LiNO_3$. The resolution is unsatisfactory for the dextran sulphates with very high molecular weight. It is not possible to clearly distinguish between 500,000 Da and 1,000,000 Dalton. The low MW dextran sulphate showed a shorter retention time than the corresponding dextranes. This might be due to the different branching of the sulphated and non-sulphated compounds.

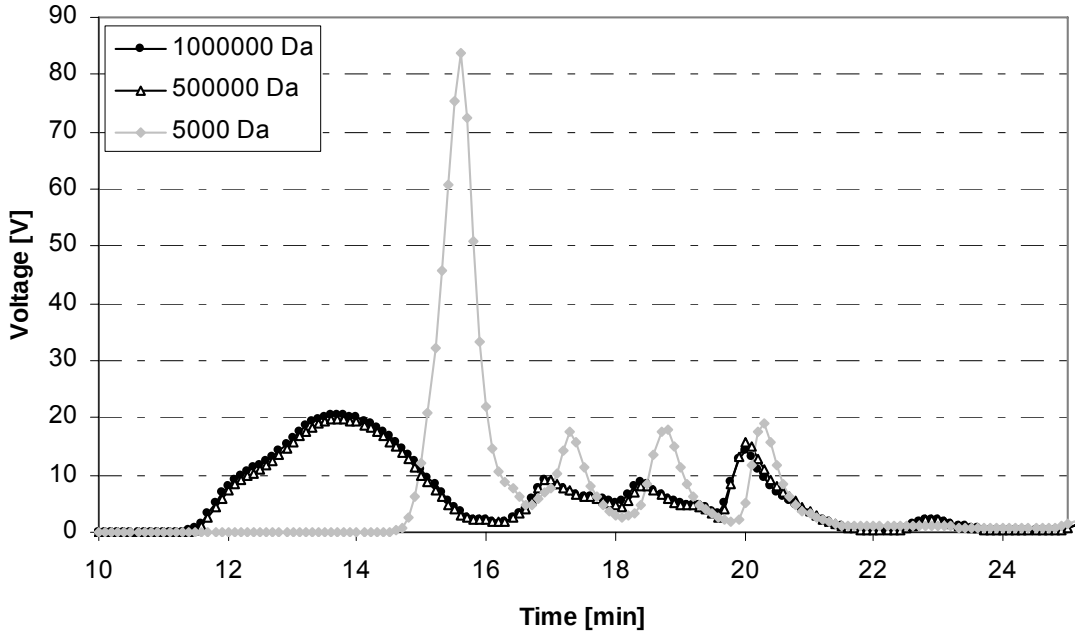



**Figure 41: Different molecular weight dextran sulphates dissolved in 0.01M $LiNO_3$, flow rate 1ml/min, concentration 2g/l**

Figure 42 shows a calibration of different amounts of Fucoidan Sigma dissolved in 0.01 M $LiNO_3$. Very little amounts of Fucoidan Sigma could not be detected in the SE-HPLC measurements and very high amounts of Fucoidan Sigma showed a different migration pattern than smaller amounts. It is not possible to directly compare the Fucoidan Sigma measurements with the dextran calibration as each polymer has its own size to molecular weight relationship.

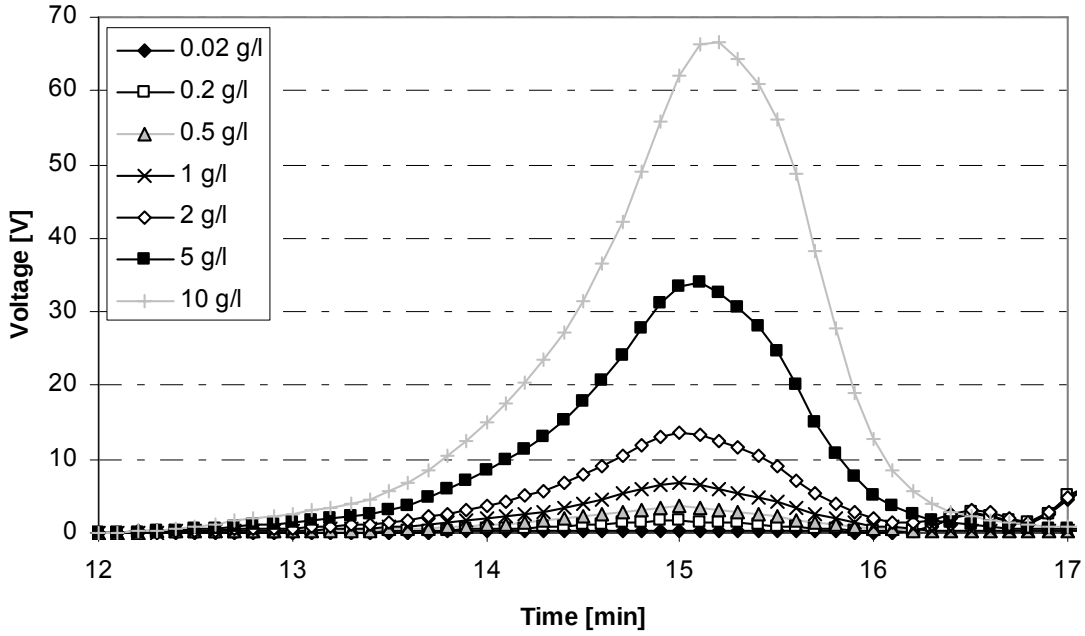



**Figure 42: Different amounts of Fucoidan Sigma (MW 300 kDa) dissolved in 0.01 M $LiNO_3$, flow rate 1ml/min**

Figure 43 shows a calibration of different amounts of heparin. Very high amounts of heparin showed a different migration pattern than smaller amounts. Very little amounts of heparin were difficult to be detected. Retention time seemed to be prolonged by higher amounts of the polysaccharide.

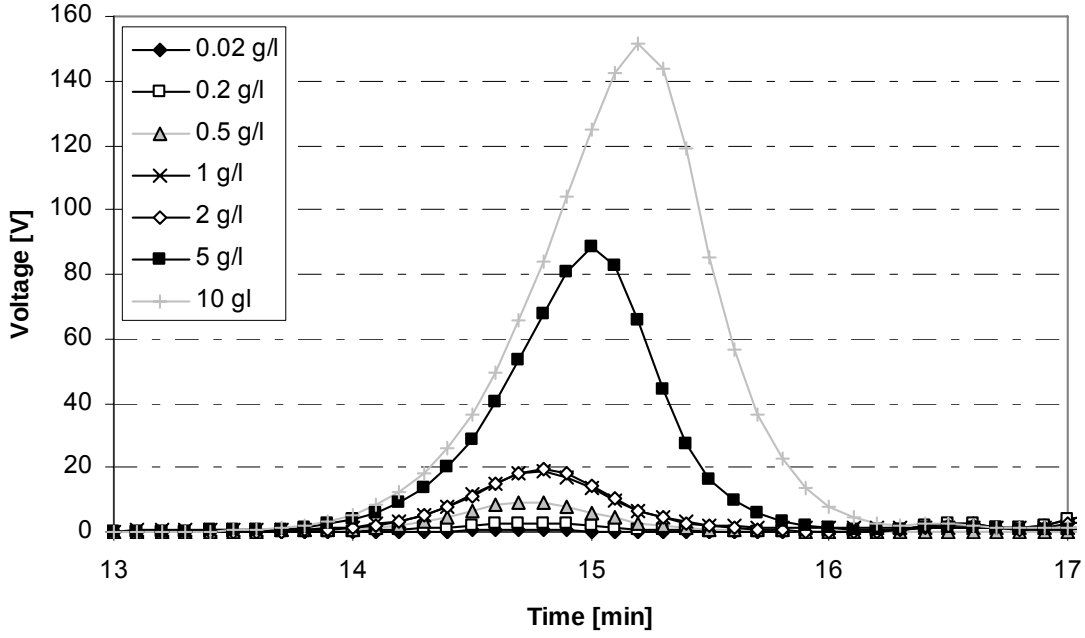



**Figure 43: Different amounts of heparin (MW 15 kDa) dissolved in 0.01 M $LiNO_3$, flow rate 1ml/min**

Calculations of the given values for the different amounts of Fucoidan Sigma and heparin revealed, that the detection of the given SE-HPLC columns was very well. The calculated area for the different amounts correlated 100% with the one detected. The $R^2$ value for the linear regression curve was 1 (data not shown).

As the distinction of the exact molecular weight was very difficult with only a refractive index detector, lacking an appropriate standard, another detection attempt was performed with a light scattering detector (DAWN DSP; wavelength 638 nm). With this system the molecular weight of polysaccharide can be determined (Granath and Flodin, 1961; Wu, 1993). The same columns were used. Evaluation was done by special software (ASTRA for Windows 4.73.04). Figure 44 shows, how the combination of light scattering detector and refractive index detector elucidated the high molecular compounds of the extracted polysaccharides. Due to physical reasons only high molecular weight molecules can be detected by light scattering (higher than 200,000 Da). The refractive index detector detects all molecules. By matching these two measurements one can see the high molecular weight molecules and their fraction of the whole mixture. When the so called refractive index increment (dn/dc) is known, exact molecular weights can be determined by light scattering analyses according to Equation 3. For fucoidan literature provided slightly different values between 0.145 and 0.155 (Fleury and Lahaye, 1993; Hemmingson et al., 2006; Theisen et al., 2000). For our calculations, a value of 0.155 ml/g was applied. The bigger fraction (>100 kDa) consisted of three subunits of which the biggest one was around 1,300 kDa. FVEhigh was further on referred to 1,300 kDa, since the amount of the fraction showing this size was exceeding the other two fractions. To get even more accurate results the three subunits had to be separated by filtration. All analysis performed on FVEhigh in this thesis were performed with the three subunits combined. If another dn/dc value would have been applied for the calculations, the determined molecular weight would differ. This has to be taken into consideration, when the molecular weight distribution of the self extracted fucoidans is discussed.

$$\text{Light scattering signal} = \sim (dn/dc)^2 \cdot \text{molecular weight} \cdot \text{concentration}$$
$$\text{Refractive index signal} = \sim dn/dc \cdot \text{concentration}$$

**Equation 3: Influence of the molecular weight on the light scattering and refractive index signal in size exclusion measurements modified after (Tartsch and Heinzmann, 2007).**

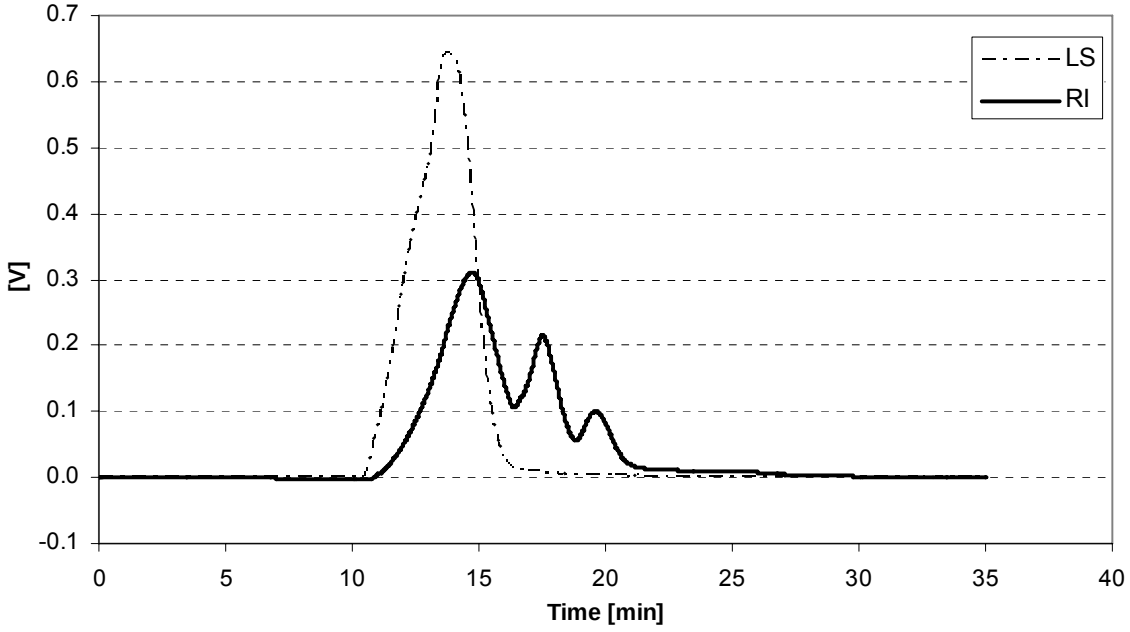



**Figure 44: FVEhigh SEC Size Determination Measurements; LS = Detected With Light Scattering Detector; RI = Detected With Refractive Index Detector**

For the smaller fraction 30 to 50 kDa was estimated (data not shown). Fucoidan Sigma was estimated to 300 kDa in size (data not shown). Figure 45 gives an overview of the approximate size determination of sulphated polysaccharides.

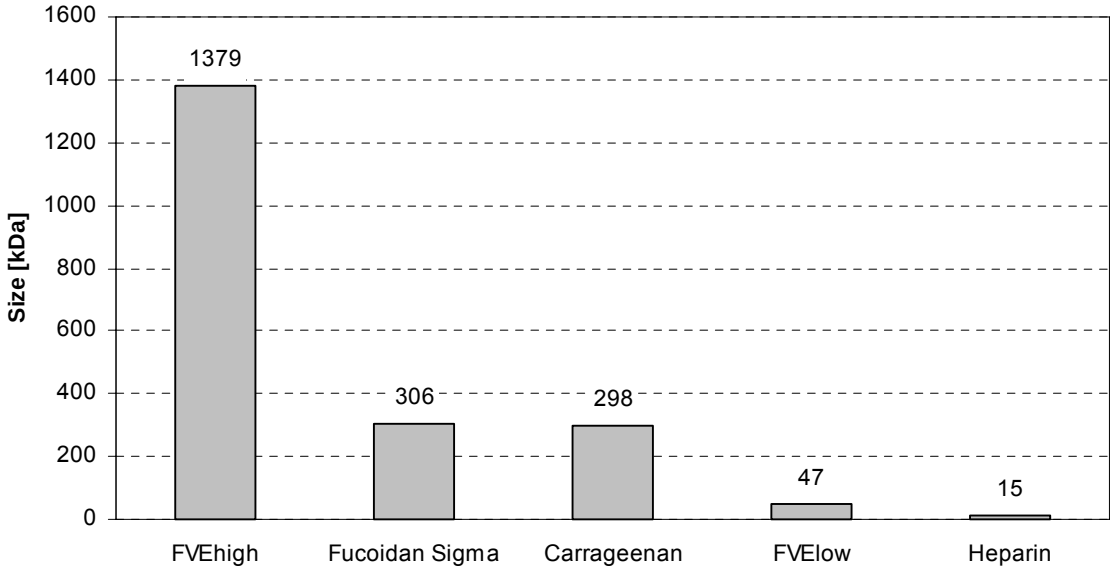



**Figure 45: Size Distribution of the analysed sulphated polysaccharides (average values)**

## 4.1.8 Modification Experiments

### 4.1.8.1 Physical Degradation

One possibility to physically degrade polysaccharides is to use ultrasound (Kulicke et al., 1993; Lii et al., 1999; Tabata et al., 1981; Zhou and Ma, 2006). No degradation could be detected by thin layer chromatography (TLC) and SE-HPLC after three hours real time acoustic irradiation using either 150 Watt and 300 Watt. No difference could be seen between the different extracts. It is possible that the glycosidic bonds in the fucoidan are too strong to be degraded by ultrasound. Another more likely possibility is that TLC and SE-HPLC detection methods were not sensible enough, as measurements done by our cooperation partners showed degradation by ultrasonic devices with similar energy inputs.

### 4.1.8.2 Chemical Degradation

Chemical degradation was very successful. It was possible with these experiments to show the benefit of the different detection methods developed during this doctoral thesis. It was possible to partially degrade the FVEs without breaking every sugar bond. As the smaller sugars still can be detected in the C-PAGE method, this reveals that not all sulphate bonds were cleaved either, as there would be no migration in the gel without the charge resulting from the sulphate bonds. This was proven by trying to migrate laminarin and dextran (data not shown) in the C-PAGE which was not possible as there are no charged groups in these molecules. Migration of dextran sulphate could be conducted very well as expected (data not shown). Figure 46 shows two fucoidan samples. In bold Fucoidan Sigma and as a thin line the same fucoidan after a chemical degradation procedure.

Another method for detecting chemical hydrolysis is the detection of reducing sugars by colorimetric methods. Figure 47 shows a DNS-analysis of the degradation of Fucoidan Sigma. The amount of reducing sugar ends was increasing with progressing hydrolysis. After 120h no more free reducing ends were added. At this point chemical hydrolysis seemed to be complete.

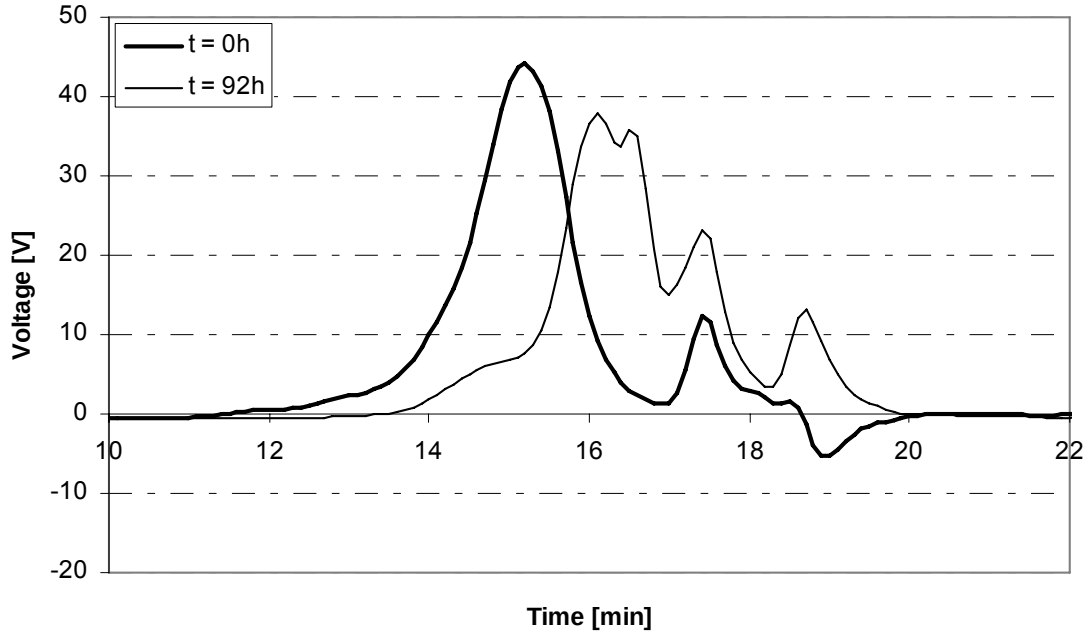



**Figure 46: Fucoidan Sigma: mild chemical hydrolysis; SEC results; bold = Sigma Fucoidan t = 0h, thin = Sigma Fucoidan degraded after 92h**

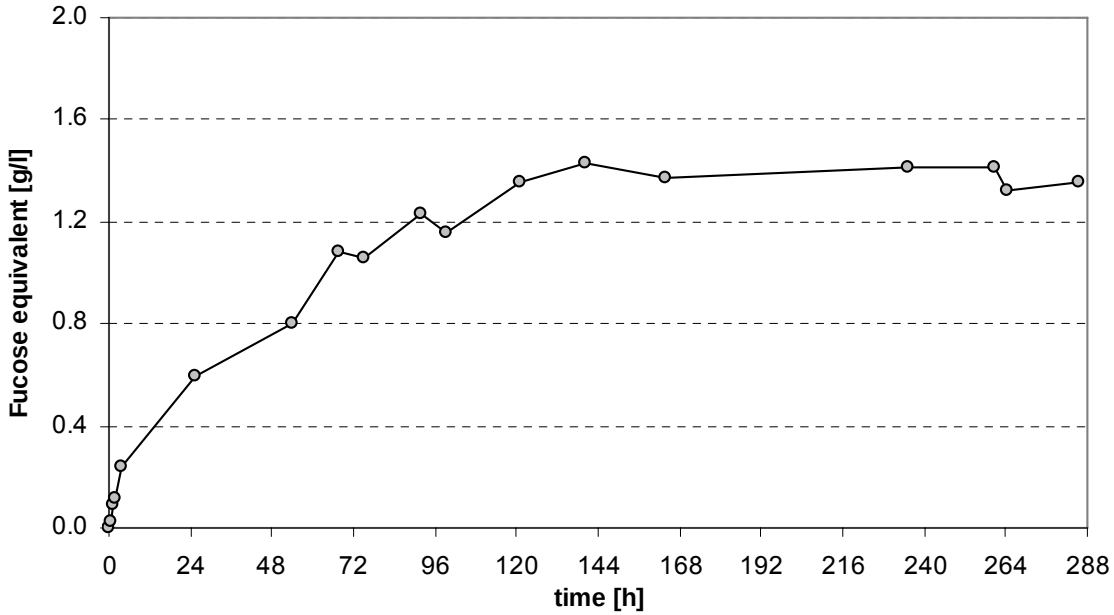



**Figure 47: Fucoidan Sigma: DNS analysis after chemical hydrolysis**

It is shown in Figure 48 that the self extracted fucoidan can be chemically degraded. After 48h the degradation is not completed down to monosaccharides but to oligosaccharides. The size of these sugars could not be determined exactly due to the lack of an appropriate standard and thus has to be roughly estimated. Figure 49 shows an Alcian Blue-stained C-PAGE gel of the chemical degradation. It can be seen very clearly how the FVEhigh molecules were cleaved and smaller (still sulphated) molecules were produced. After 48h (lane 8) a very large amount of small molecules became evident. These molecules were faster migrating than the brom phenolic blue in the running buffer.

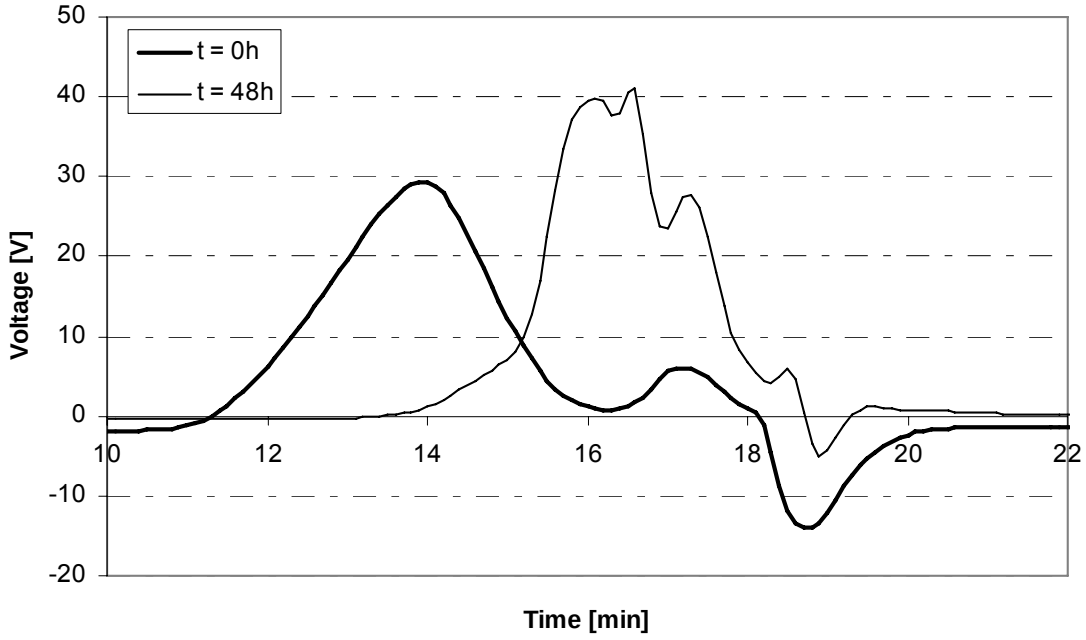



**Figure 48: FVEhigh: mild chemical hydrolysis; SEC results; bold = FVEhigh t = 0; thin = FVEhigh degraded after 48h**

Figure 50 shows the same gel after the silver nitrate staining. The existence of the smaller sulphated saccharides became even more evident as the staining procedure is more sensitive.

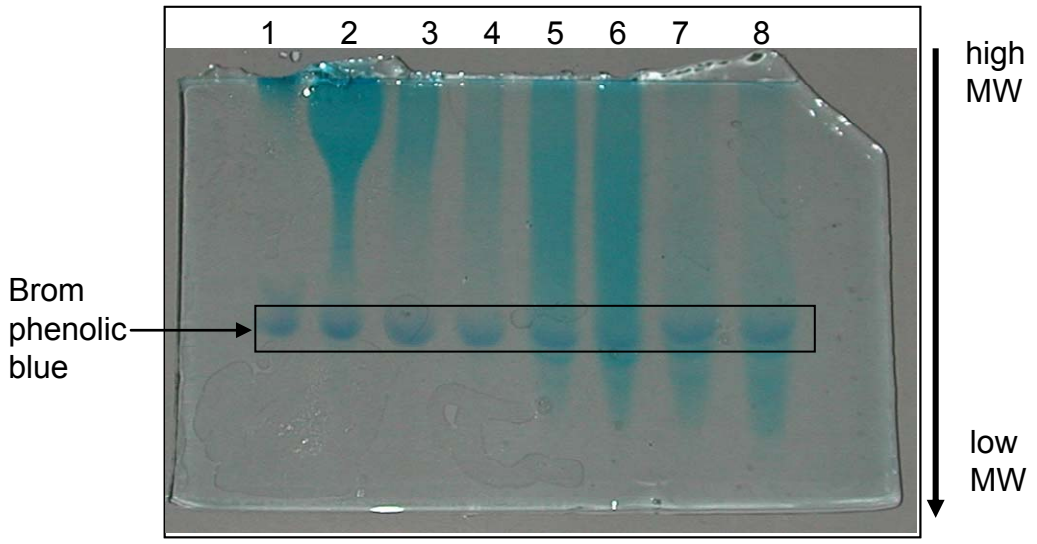



**Figure 49: FVEhigh: Alcian Blue stained C-PAGE gel of chemical degradation; 1 = 0h; 2 = 1h; 3 = 2h; 4 = 4h; 5 = 6h; 6 = 8h; 7 = 24h; 8 = 48h**

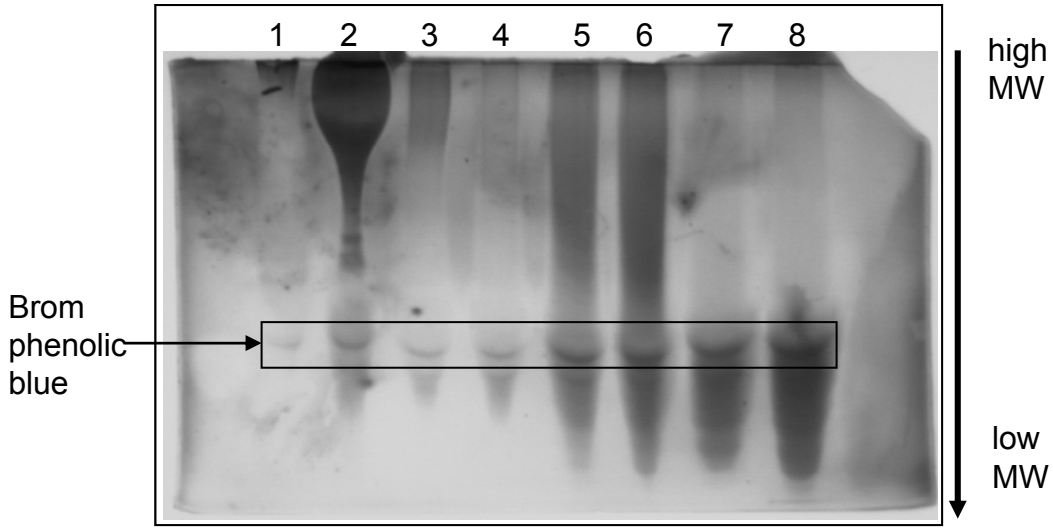



**Figure 50: FVEhigh: silver nitrate stained C-PAGE gel of chemical degradation; 1 = 0h; 2 = 1h; 3 = 2h; 4 = 4h; 5 = 6h; 6 = 8h; 7 = 24h; 8 = 48h**

#### 4.1.8.3 Enzymatic Degradation

The enzymatic degradation of FVEhigh, LDEhigh and Fucoidan Sigma was performed with different commercial enzymes, supernatants and cell disruption solutions of cultivated microorganisms.

It was not possible to produce and collect high amounts of the degraded fucoidans (oligofucoidans) that could then be analysed for their composition and structure. Information on the production of reducing ends or smaller polysaccharides can be seen in the section about microorganisms and commercial enzymes (chapter 4.3) since this answers the question whether there is a general ability to degrade fucoidan or not.

### 4.1.9 Summary – Results on Sulphated Polysaccharides and Oligosaccharides

During the project quite high amounts of fucoidan from *Fucus vesiculosus* could be produced, considering that the yield never reached a value higher than 1%. With this self-extracted fucoidan several experiments could be performed. In addition to that, LDEhigh was produced and could be used in some other experiments as well. To compare the different results commercial fucoidan from *Fucus vesiculosus* (Sigma) was used as an independent source.

Extraction and filtration (Vivaflow) gave two raw extracts. These extracts were dialysed (Visking® dialysis tubing, regenerated cellulose; 12-14 kDa, pore diameter 25 Å) and then analysed by size exclusion chromatography, C-PAGE and colorimetric methods.

Further processing lead to two different fractions, the larger one (FVEhigh) with a molecular weight of 1,300 kDa and the smaller one with a molecular weight between 30 to 50 kDa. FVEhigh was more strongly sulphated than the smaller fragments. The amount of FVEhigh exceeded FVElow by an order of magnitude.

The amount of the fucoidan produced is still quite low in order to guarantee the same high quality in every experiment performed. No physical degradation could be detected, whereas chemical degradation could be monitored successfully.

## 4.2 Bioactivity

### 4.2.1 Anti-coagulant Activity

For different substances the coagulation time was tested with the Hepato-Quick Test. As a control, sole citrate buffer was used. To determine the statistical error of the coagulometer, citrate buffer was measured in each experiment ("no substance"). The blood used in these experiments was taken from mice by Dr. Franz Vauti, Institute of Biochemistry and Biotechnology, Department of Molecular Biology, TU Braunschweig. In Figure 51 different polysaccharides and their coagulation time are compared.

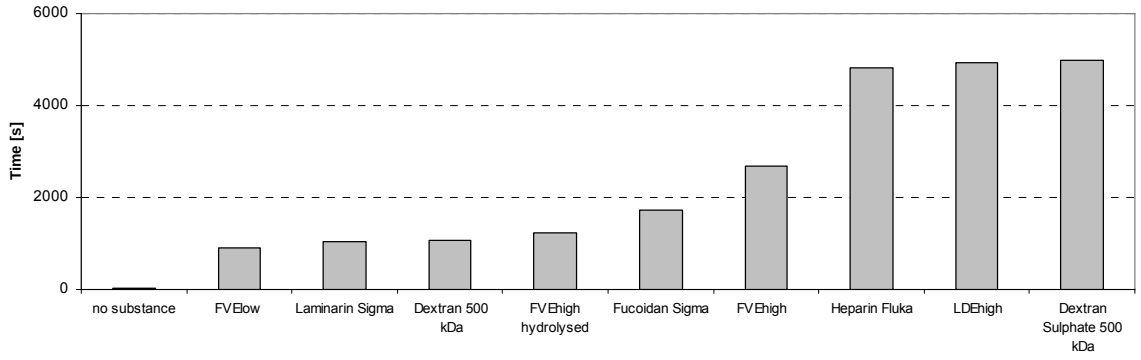



**Figure 51: Influence of various polysaccharides on the coagulation time of blood (mice); Hepato-Quick test**

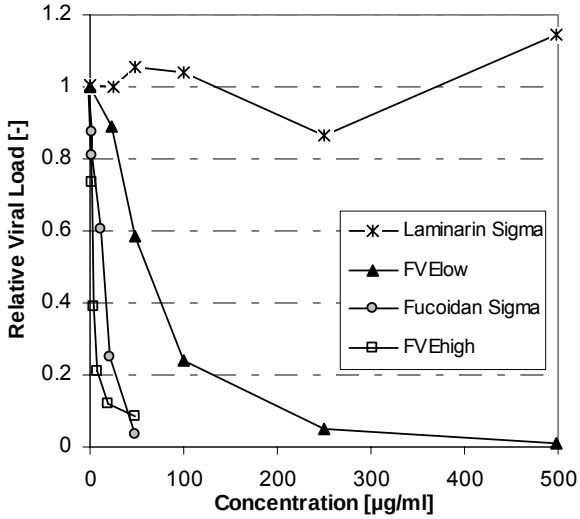
Several compounds produced by different extraction procedures were tested. It became evident that FVElow does not influence the clotting time as much as the bigger molecules do. The bigger fucoidans showed the best results. Some of them proved even better than the commercially available heparin that is used as an anti-clotting agent in coagulation therapy.

Despite of these results the tests have to be redone in order to get more parameters to compare. The value with no substance at all was measured eight times and was reproducible very well (+/-0.5 sec). The other values fluctuated. In order to get reliable results several other substance concentrations should be measured.

### 4.2.2 Anti-viral Activity

Anti-viral activity was tested by Stefanie Thulke from the Charité-Universitätsmedizin Berlin, CCM – Medizinische Klinik m.S. Onkologie/Hämatologie, Charitéplatz , 10117 Berlin, Germany, in collaboration with Yvonne Naumann from the University of Erlangen-

Nuremberg as this group has been intensively working with viruses (Nitsche et al., 2004; Nitsche et al., 2003; Radonic et al., 2005a; Radonic et al., 2005b; Radonic et al., 2004; Rechter et al., 2006; Thulke et al., 2006). Figure 52 shows the anti-viral activity of FVEhigh and FVElow as well as for Fucoidan Sigma. Laminarin without sulphate groups has been taken as a negative control.




| | $IC_{50}$ [µg/ml] |
|---|---|
| Laminarin Sigma | >500 |
| FVElow | 64 +/- 24 |
| Fucoidan Sigma | 13.3 (+3.7 / -5.3) |
| FVEhigh | 4.4 +/- 1.9 |

**Figure 52: Anti-viral tests: Influence on the relative viral load of different polysaccharides; $IC_{50}$ values for marine polysaccharides**

The activity of the four polysaccharides against HCMV was tested. Even at concentrations as low as 4 µg/ml FVEhigh showed a very good anti-viral activity. Fucoidan Sigma also had slightly better anti-virus potential than the commercial ganciclovir (around 14 µg/ml). No effect could be seen with a laminarin treatment. FVElow also showed an effect on the virus.

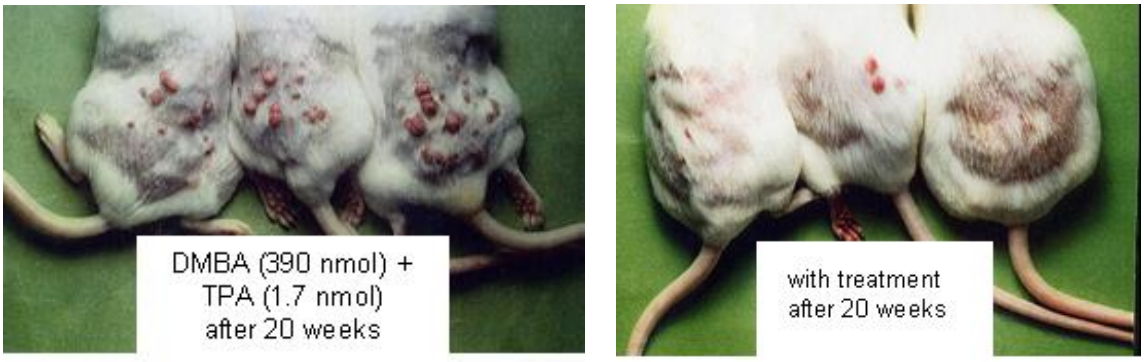
### 4.2.3 Anti-tumoral Activity

The study on the anti-tumoral activities of the different extracts was performed by Prof. Harukuni Tokuda at the Prefectural University of Medicine in Kyoto, Japan. Several samples of self-extracted and commercially available fucoidans were examined in these experiments.

#### 4.2.3.1 *In vivo* two-Stage Mouse Skin Carcinogenesis Tests

##### *4.2.3.1.1 DMBA/TPA-induced*

To visualise the effect of anti-tumour agents, Figure 53 shows the pictures of mice treated with the tumour-inducing DMBA and promoted by TPA and mice treated with an anti-tumour agent. It is obvious that the number of developed tumours was much less with the anti-tumour agent.




**A** **B**

**Figure 53: Inhibition of DMBA/TPA-induced tumour promotion by multiple application of an anti-tumour agent. All mice were carcinogenically initiated with DMBA (390 nmol) and promoted with TPA (1.7 nmol) given twice a week; A = untreated mice; B = mice displaying less tumours (successful cancer inhibition)**

Figure 54 and Figure 55 show the comparison between the anti-tumour capacity of the different compounds after the tumour induction through DMBA/TPA. Fucoidan Sigma showed the highest anti-tumour ability. FVEhigh was only slightly more potent than FVElow. After 7 weeks, the positive control reached 100%, whereas the fucoidan samples did not reach 100% until after 20 weeks of experiments.

The average number of papillomas/mouse can be seen in Figure 55. The number of papillomas/mouse was between 7 and 9 after 20 weeks of analyses. Mice fed with Fucoidan Sigma displayed two papillomas less than untreated mice. Mice treated with either FVEhigh or FVElow only had one papilloma less.

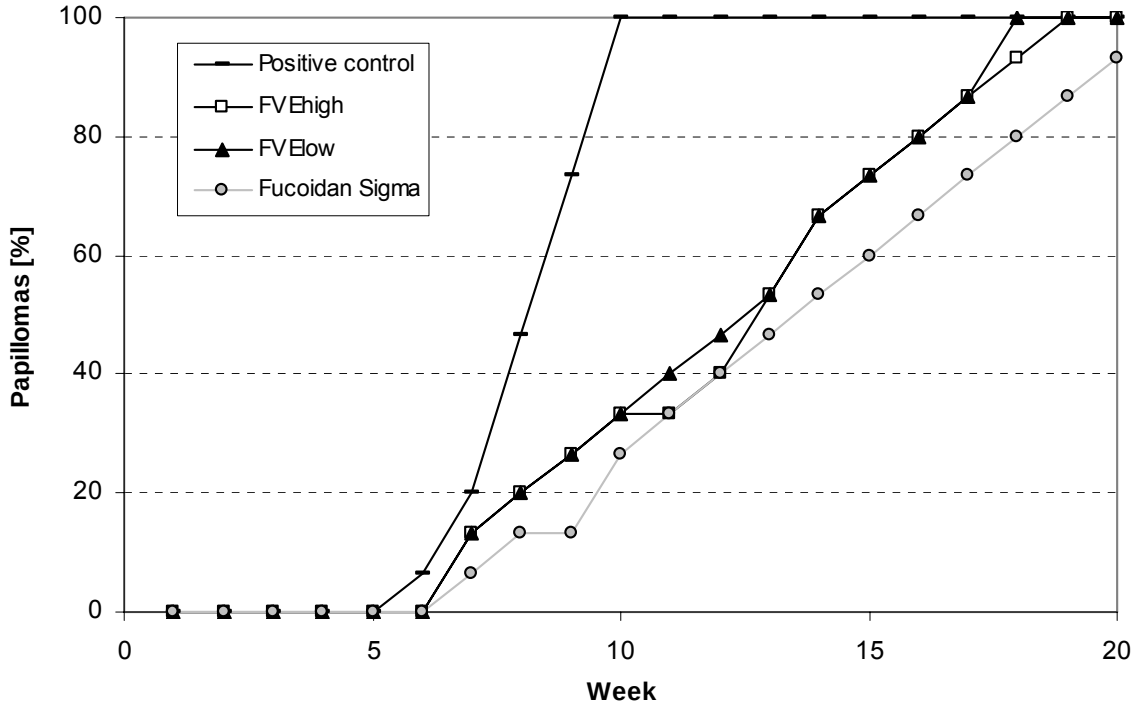



**Figure 54: Inhibition of DMBA/TPA-induced tumour promotion by multiple application of fucoidan. All mice were carcinogenically initiated with DMBA (390 nmol) and promoted with TPA (1.7 nmol) given twice a week; Percentage of mice bearing papillomas**

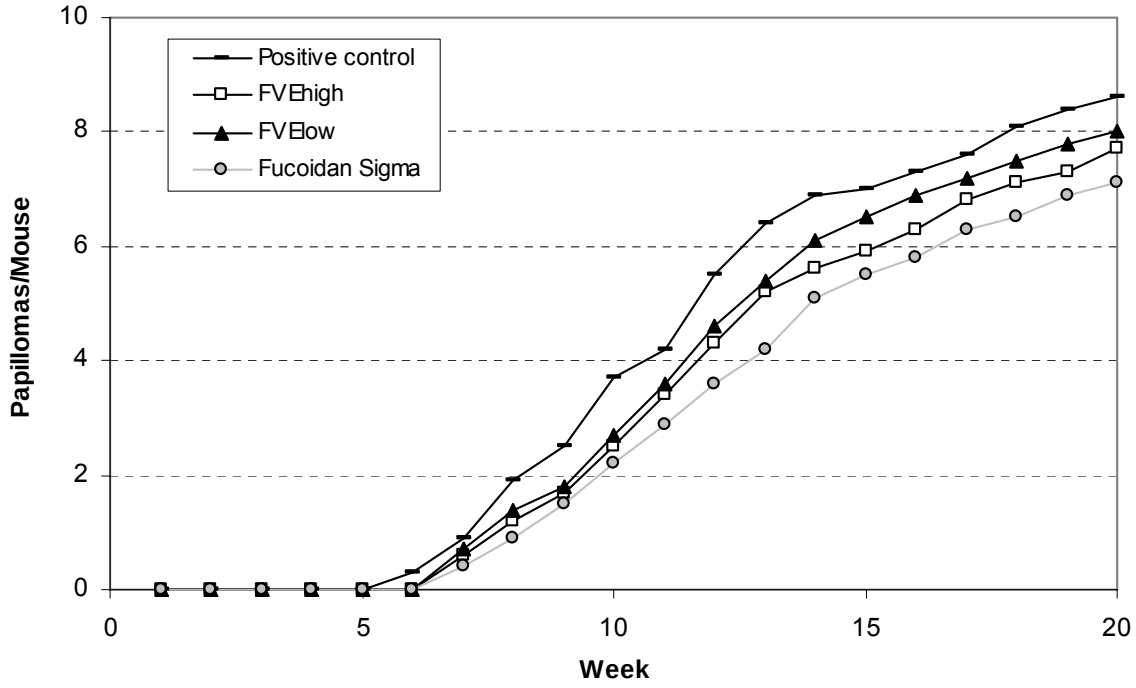



**Figure 55: Inhibition of DMBA/TPA-induced tumour promotion by multiple application of fucoidan. All mice were carcinogenically initiated with DMBA (390 nmol) and promoted with TPA (1.7 nmol) given twice a week; Average number of papillomas per mouse**

#### *4.2.3.1.2 Peroxynitrite/TPA-induced*

Figure 56 and Figure 57 show the inhibition of Peroxynitrite/TPA-induced skin cancer by FVEhigh. As a reference the anti-inflammatory agent curcumin is plotted.

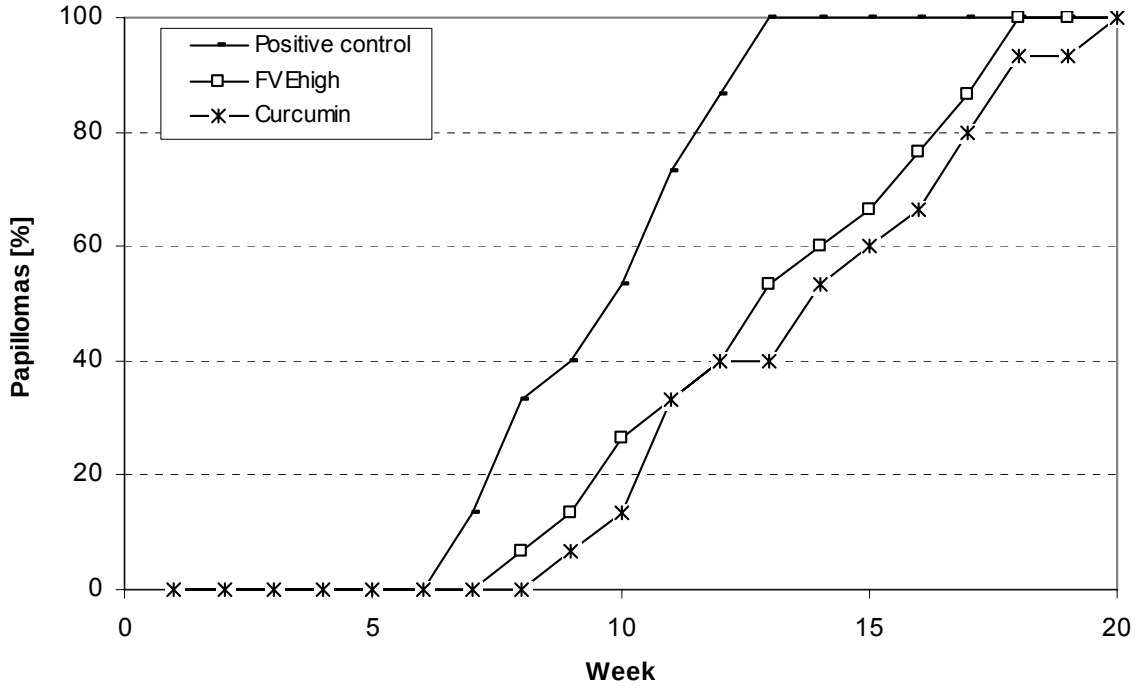



**Figure 56: Inhibition of Peroxynitrite/TPA-induced tumour promotion by multiple application of FVEhigh. All mice were carcinogenically initiated with Peroxynitrite (390 nmol) and promoted with TPA (1.7 nmol) given twice a week; Percentage of mice bearing papillomas.**

As it can be seen in Figure 57 FVEhigh successfully inhibits the Peroxynitrite/TPA-induced tumour promotion. After 20 weeks, only four tumours were promoted per mouse. By contrast, untreated mice developed around seven tumours. This results in a tumour reduction rate of around 43%.

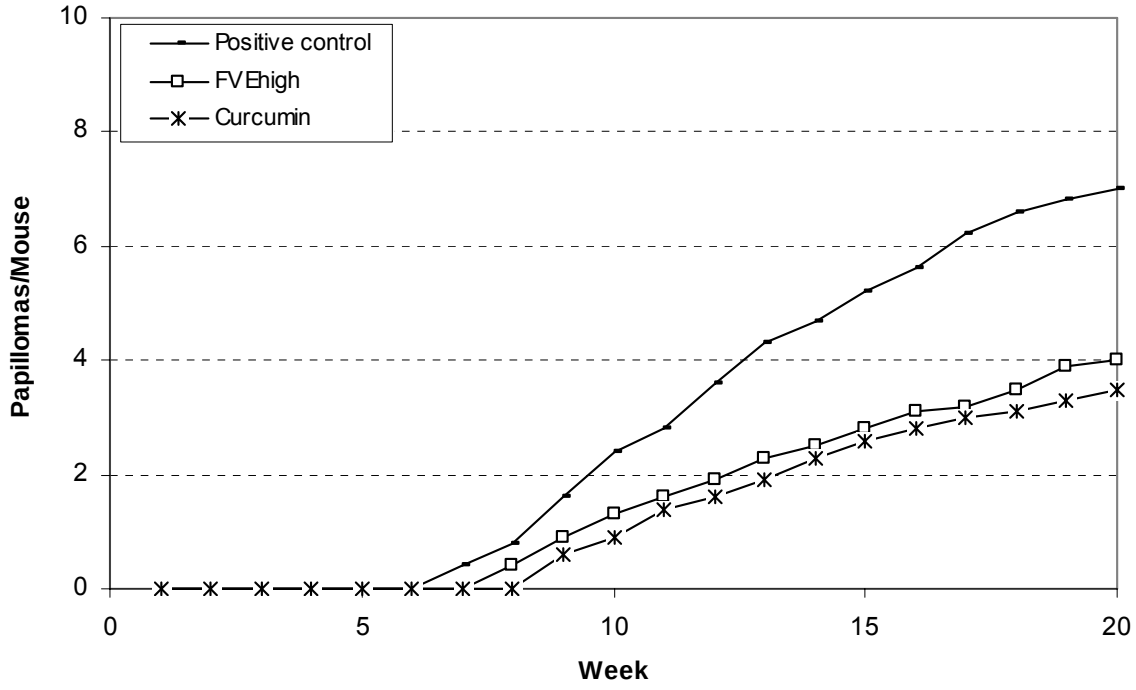



**Figure 57: Inhibition of Peroxynitrite/TPA-induced tumour promotion by multiple application of FVEhigh. All mice were carcinogenically initiated with Peroxynitrite (390 nmol) and promoted with TPA (1.7 nmol) given twice a week; Average number of papillomas per mouse**

#### 4.2.3.2 Short term *in vitro* Bioassay for the Inhibition of Epstein Barr Virus Early Antigen (EBV-EA) Activation Induced by TPA

Four different fucoidans were tested for their ability to inhibit the Epstein Barr Virus early antigen activation induced by TPA. Different TPA/compound ratios were applied. Table 12 gives the relative ratio of the compound activation with respect to the positive control. The values in parentheses represent the percentage of viable cells.

As Table 12 shows, the high molecular weight substances like Fucoidan Sigma and FVEhigh showed an activity potency. The LDEhigh also displayed potency against TPA induction of the EBV-EA. FVElow had the lowest influence on tumour formation.

**Table 12: Inhibitory effects of high-molecular weight compounds on TPA-induced EBV-EA activation**

| | Concentration (mol ratio/TPA)** | | | |
|---|---|---|---|---|
| | 1000 | 500 | 100 | 10 |
| Compound | % to control (% viability)* | | | |
| Fucoidan Sigma | 8.0 ± 0.2 (60) | 42.1 ± 1.4 | 71.2 ± 2.2 | 92.8 ± 0.7 |
| FVEhigh | 10.3 ± 0.3 (60) | 44.4 ± 1.5 | 72.1 ± 2.1 | 98.8 ± 0.7 |
| FVElow | 14.9 ± 0.5 (60) | 53.9 ± 1.6 | 78.5 ± 2.1 | 100.0 ± 0.5 |
| LDEhigh | 11.4 ± 0.5 (60) | 50.7 ± 1.4 | 75.2 ± 2.0 | 100.0 ± 0.4 |

*Values are given for EBV-EA activation (%) in the presence of the test compound relative to the control (100%). The numbers in parentheses are the viability of the tested Raji-cells.
**Activation was attained by treatment with TPA (32pmol). The tested polysaccharides were applied in multiples of this concentration (10-1000 fold).

## 4.2.4 Antibodies Against Fucoidan

Panning was conducted with Fucoidan Sigma as target molecule. After the panning one potent binder could be isolated (H8; see Figure 58). Subsequently the scFv were produced in *E.coli* and their cross-binding reactions were checked. No other extract (high, low) or dextran sulphate could be bound to the produced antibody (data not shown).

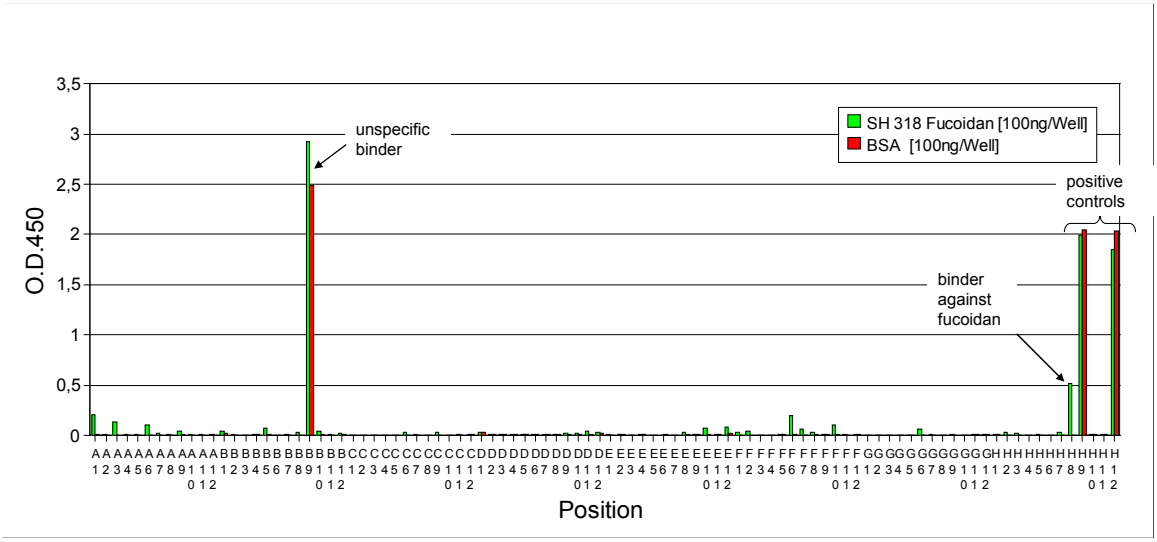



**Figure 58: Monoclonal ELISA signals for fucoidan antibody; red = signals evoked by BSA; green = signals evoked by potential fucoidan binders**

Antibody clone SH318-H8 developed binding against Fucoidan Sigma, but no binding against BSA. Therefore this antibody seemed to be a potent candidate. Gene sequencing analysis gave the following segments:

Variable heavy chain:
HV: IGHV3-73*01
D: IGHD4-17*01
HJ: IGHJ1*01

Variable light chain:
LV: IGLV7-43*01
LJ: IGLJ3*02

### 4.2.5 Summary – Results on Bioactivity of Fucoidan

Several potent bioactivities of fucoidan were tested. Anti-viral activities of FVEhigh and Fucoidan Sigma were very high. FVElow showed an anti-viral activity, but much lower than for the other two extracts. Laminarin did not show any anti-viral activity against HCMV. All applied sulphated polysaccharides showed an anti-tumour activity against TPA induced skin cancer. The anti-tumour activity of Fucoidan Sigma was higher than for FVEhigh, FVElow and LDEhigh. Anti-coagulant tests revealed that high molecular weight fucoidans prolonged the blood clotting time, with LDEhigh showing the highest activity. It was possible to find an antibody against Fucoidan Sigma. The binding was not very strong, but specific for Fucoidan Sigma.

## 4.3 Studies on the Fucoidan-Degrading Potential of Various Microorganisms

**General Information**

In 2005 the project started under the premises of already having a fungus (*Dendryphiella arenaria* TM 94) that was able to degrade fucoidan from *Laminaria digitata,* as published by Wu et al. (Wu et al., 2002). First cultivations on solid state media were conducted according to the descriptions of Wu (Wu et al., 2002). Unfortunately it was not possible to get any reasonable results with this cultivation method because of the huge amount of monosaccharides in the cultivation media (wheat bran, straw). A number of detection methods were used in order to neglect this high background signals. With none of these methods any reasonable fucoidan or laminarin degradation could be detected.

New liquid cultivation forms had thus to be found as well as detection methods for the reducing sugars produced during hydrolysis of the fucoidan. The detection of a strain that could act as a positive control was a very important step, as the project was lacking it at the time. Isolation experiments, literature research for other strains and method developments concerning detection and cultivation followed. *Dendryphiella arenaria* TM 94 was cultivated at a scale of up to 8 l to gain more information about the strain. The other strains presented in this work are the result of an intense literature work in 2006 to get a suitable strain for the experiments.

**Concept**

The idea was that no microorganisms were present in the enzymatic degradation experiments. One has to distinguish between the cultivation experiments (whole cell conversion, leading to biomass production (consumption of the C-source) which can be detected gravimetrically) and the cell-free environment (enzymatic degradation, leading to smaller fragments of the sulphated polysaccharides which can be detected). For the enzymatic degradation experiments the strains were cultivated in either a minimal medium with the polysaccharide that was to be degraded, or in an induced (with polysaccharide) or non-induced (without any additional C-source) full medium that was established to produce high amounts of cell mass. The cells were either discarded or disrupted and the supernatant or the cell disruption solution (without debris) were used as the enzyme solution.

### 4.3.1 *Dendryphiella arenaria* TM 94

As this was the first organism available, many experiments were done with this strain. First cultivations on solid state media were conducted according to the descriptions of Wu (Wu et al., 2002). It was not possible to get any reasonable results with this cultivation method because of the huge amount of monosaccharides in the cultivation media (wheat bran, straw). A number of detection methods were used in order to neglect these high background signals. With none of these methods any reasonable fucoidan or laminarin degradation could be detected. All results shown emanate from liquid cultivations and are thus comparable to the other strains cultivated. Liquid cultivation was established in order to keep the possibility of scaling up the whole process.

**Cultivation experiments**

For *Dendryphiella arenaria* TM 94 biomass production can be enhanced by a factor of five by applying optimal conditions. The maximum dry mass of *Dendryphiella arenaria* TM 94 measured in this thesis was around 18 g/l and was produced in a 250ml shake flask with complex medium and 20g/l supplemented glucose. Measuring faults might have occurred due to precipitating salts. *Dendryphiella arenaria* TM 94 formed pellet structures as common for filamentous fungi (Munk, 2001) as well as a high spore production which resulted in a deep black colouring of the medium.

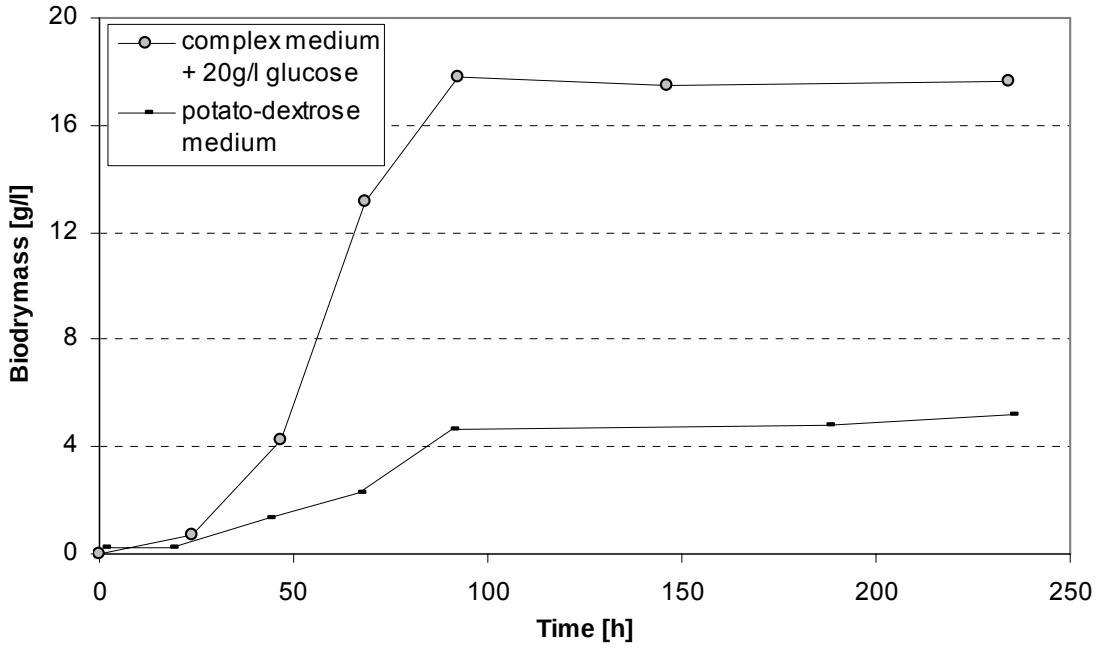



**Figure 59: *Dendryphiella arenaria* TM 94: Cultivation in 250 ml shake flasks, 27 °C, fungi medium 1 supplemented with 20g/l glucose and potato-dextrose medium non-supplemented. With optimised conditions, biomass production can be enhanced.**

After successful optimisation of the biomass production, the cultivation procedure was scaled up to 8l working volume in a Bioreactor (Biostat E Braun, Melsungen). Figure 60 shows the record of the $pO_2$-development and biomass production during the cultivation of *Dendryphiella arenaria* TM 94. The stirrer speed was manually adjusted to assure a sufficient oxygen supply. The decrease in the $pO_2$-development correlated very well with the increase in biomass. Measurements of the glucose consumption and the pH (data not shown) indicated that all glucose was consumed after around 70h. After this time, the fungus started to consume the more complex resources in the medium, as biomass was still increasing and the pH was raising (due to the release of free ammonium-ions of the complex media components).

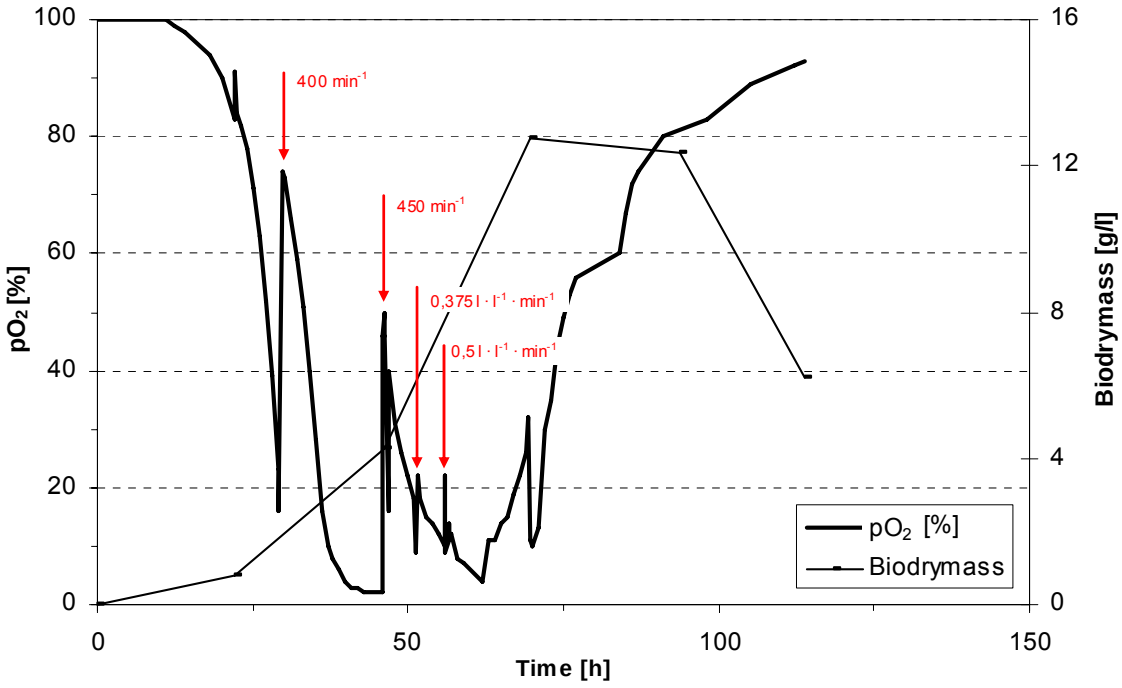



**Figure 60: *Dendryphiella arenaria* TM 94: Bioreactor cultivation of 8l , 300 $min^{-1}$, 0.25 $l \cdot l^{-1} \cdot min^{-1}$, 27 °C, start pH 5.4, end pH 8.1; fungi medium 1 with 10g/l glucose**

Several other C-sources were tested in shake flasks accordingly. Table 13 shows the results of cultivation experiments of *Dendryphiella arenaria* TM 94. It can be seen that *Dendryphiella arenaria* was able to metabolise several monosugars and starch very well. Dextrans seemed to be much harder to degrade.

**Table 13: *Dendryphiella arenaria* TM 94: Cultivation experiments on minimal medium; +++ = very good growth; ++ = good growth; + = slight growth; - = no growth**

| C-source | Growth |
|---|---|
| Complex medium | +++ |
| D-Glucose (2g/l) | ++ |
| D-Galactose | +++ |
| D-Xylose | +++ |
| D-Sucrose | ++ |
| D-Raffinose | ++ |
| FVEhigh | +/- |
| Fucoidan Sigma | - |
| Carrageenan | + |
| Dextran T 70 | + |
| Dextran T 500 | + |
| Dextran T 2000 | + |
| Dextran T 5000 | + |
| Starch | +++ |
| *Laminaria digitata* powder | ++ |

**Enzymatic Degradation Experiments**

*Dendryphiella arenaria* TM 94 was tested for extra cellular enzyme activity according to the investigations of Wu et al. (Wu et al., 2002). Unfortunately no enzyme activity in the supernatant could be detected on any polysaccharide except for laminarin (data not shown).

### 4.3.2 Self Isolate WHV059

The fungus WHV059 was isolated from the foot of the alga *Fucus vesiculosus* in 2005. As it is an own isolate no cultivation procedure was available. Cultivation was done according to lab practice. Analyses done by Alga Zuccharo (Institute of Microbiology, TU Braunschweig) revealed that it is a fungus of the genus *Acremonium sp*. that was not equivalent to any reported strain (personal communication, (Zuccaro et al., 2004)).

**Cultivation Experiments**

*Biomass enhancement*

As it was postulated that the enzymatic production might be coupled with the biomass production, several cultivation experiments were performed aimed at an enhancement of the biomass. Figure 61 shows the growth curve of Self Isolate WHV059 with medium 1

and 30 g/l of added glucose. Biomass reached 15 g/l after approximately 98 h. With other C-sources only lower values can be achieved (data not shown). Self Isolate WHV059 was forming pellet structures as it is common for filamentous fungi (Munk, 2001). The produced spores resulted in a orange colouring of the medium.

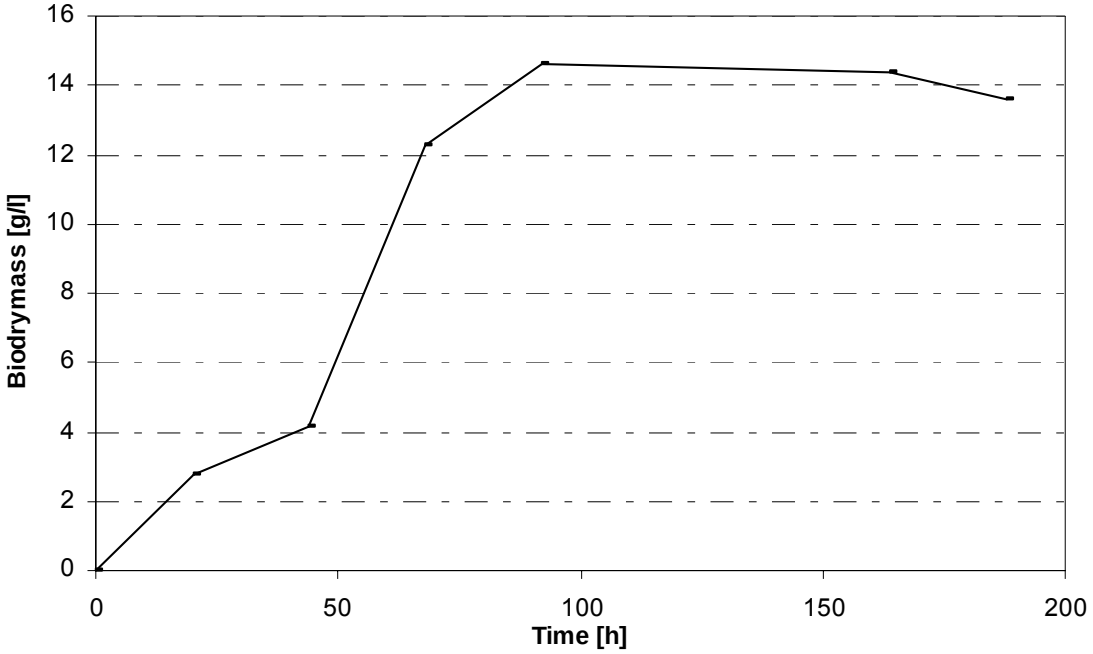



**Figure 61: Self Isolate WHV059: Growth curve for a cultivation in a shake flask (1l) in fungi medium 1 with 30 g/l added glucose; 27 °C; 100 $min^{-1}$**

*Polysaccharide Conversion*

As the wanted fucoidanase might have been part of a complex polysaccharide degrading system several attempts were performed to degrade different sulphated and non-sulphated polysaccharides. This should give a hint about whether the newly found fungus WHV059 had such a polysaccharide degrading system. Table 14 gives an overview of the cultivation experiments performed with Self Isolate WHV059.

**Table 14: Self Isolate WHV059: Cultivation experiments on minimal medium; +++ = very good growth; ++ = good growth; + = slight growth; - = no growth**

| C-source | Growth |
|---|---|
| Complex Medium | +++ |
| D-Glucose (2g/l) | ++ |
| D-Glucose (10g/l) | +++ |
| D-Glucose (20g/l) | +++ |
| D-Glucose (30g/l) | +++ |
| D-Galactose | + |
| D-Xylose | ++ |
| D-Sucrose | +++ |
| D-Raffinose | ++ |
| FVEhigh | +/- |
| Fucoidan Sigma | - |
| Carrageenan | - |
| Dextran T 70 | + |
| Dextran T 500 | + |
| Dextran T 2000 | + |
| Dextran T 5000 | + |
| Starch | + |
| *Laminaria digitata* powder | ++ |

### Enzymatic Degradation Experiments

Only a few enzymatic degradation experiments were performed on Self Isolate WHV059. The only positive results could be detected with disrupted cells. No enzyme activity could be measured with supernatant (data not shown). Figure 62 shows the enzymatic degradation experiments with several sulphated polysaccharides. It can be seen that Self Isolate WHV059 was able to cleave laminarin very well (as *Dendryphiella arenaria* TM 94 did). FVElow was also degraded slightly. A cell disruption solution of Self Isolate WHV059 did not show any activity on FVEhigh or Fucoidan Sigma.

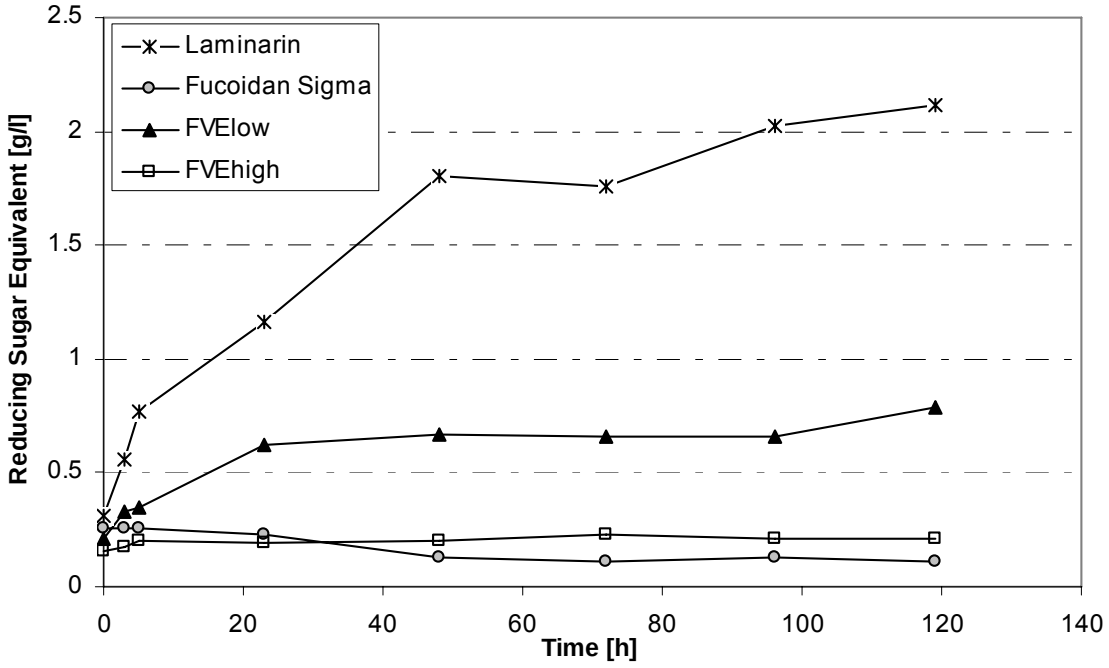



**Figure 62: Self Isolate WHV059: Enzymatic degradation of several sulphated polysaccharides by a cell disruption solution of 37 °C**

### 4.3.3 Self Isolate WHV012

The fungus WHV012 was also isolated from the foot of the alga *Fucus vesiculosus* in 2005. As it is an self isolate no cultivation procedure was available. Cultivation was thus done according to lab practice for fungi. Table 15 shows the cultivation experiments performed with Self Isolate WHV012. Enzymatic degradation experiment have not been performed for this fungus.

**Table 15: Self Isolate WHV012: Cultivation experiments on minimal medium; +++ = very good growth; ++ = good growth; + = slight growth; - = no growth**

| C-source | growth |
|---|---|
| Complex Medium | +++ |
| D-Glucose (2g/l) | + |
| D-Glucose (10g/l) | ++ |
| D-Glucose (20g/l) | +++ |
| D-Glucose (30g/l) | +++ |
| D-Galactose | + |
| D-Xylose | +++ |
| D-Sucrose | +++ |
| D-Raffinose | ++ |
| FVEhigh | +/- |
| Fucoidan Sigma | - |
| Carrageenan | - |
| Dextran T 70 | - |
| Dextran T 500 | - |
| Dextran T 2000 | - |
| Dextran T 5000 | - |
| Starch | ++ |
| *Laminaria digitata* powder | ++ |

### 4.3.4 *Saccharophagus degradans* DSM 17024

*Saccharophagus degradans* was a quite complicated microorganism to cultivate. Its ability to degrade agarose lead to decomposition of the agar plates and the microorganism thus had to be inoculated onto new agar plates very often. In addition to that the microorganism tended to loose its ability to degrade agar when cultivated on a complex medium without polysaccharides. Plated on agar plates after cultivation in liquid media the strain either lost its ability to degrade the agar plate or its eumelanin production system (no colouring of the colonies). The experiments were all done with freshly inoculated cultures in order to have the same premises.

**Cultivation Experiments**

Figure 63 shows the growth curve of *Saccharophagus degradans* in complex medium as well as in minimal medium with FVEhigh as C-source. Due to biofilm formation on the glass surface, only the absorbance is plotted. After approximately 48h a stationary stage was reached both with complex medium and with FVEhigh. The OD at 600nm, however, was considerably lower with FVEhigh.

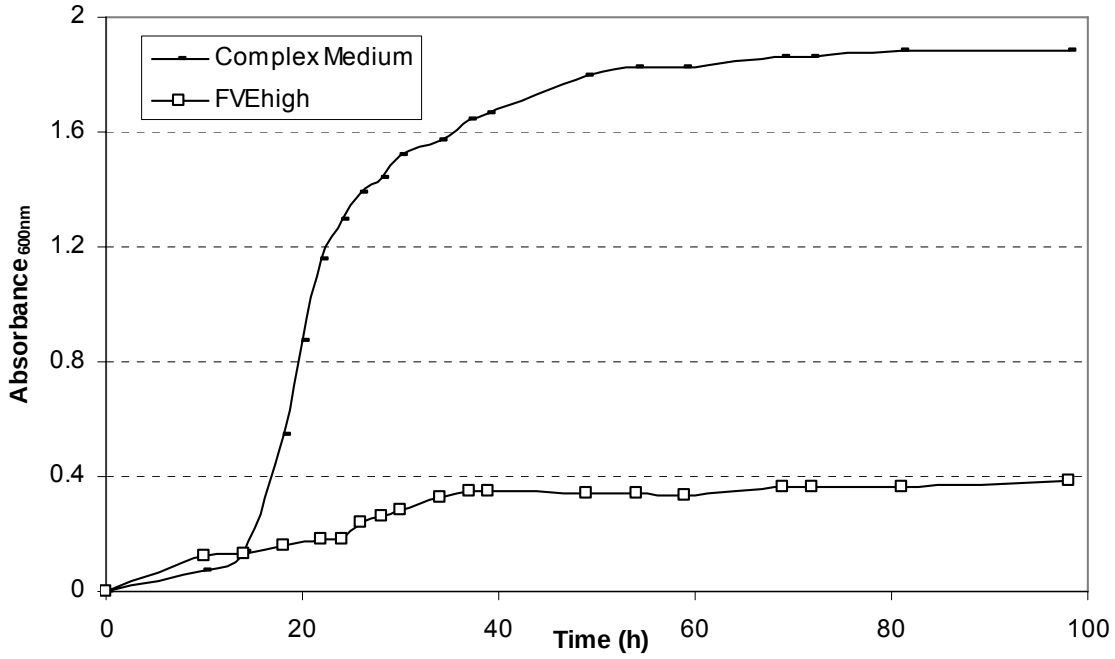



**Figure 63: *Saccharophagus degradans*: Growth curve with different C-sources in minimal medium; 27 °C**

Table 16 shows the ability of *Saccharophagus degradans* to degrade FVEhigh. The production of cell mass was understandably lower than the cell mass production with complex medium, but it seemed that *Saccharophagus degradans* was able to utilise FVEhigh as a C-source. Biomass determination was very difficult as *Saccharophagus degradans* formed a biofilm on the glass surface of the shake flask. For the absorption values, the biofilm was partly destroyed by shaking the shake flask.

**Table 16: *Saccharophagus degradans:* Cultivation experiments on minimal medium*;* +++ = very good growth; ++ = good growth; + = slight growth; - = no growth**

| C-source | growth |
|---|---|
| Complex medium | +++ |
| D-Glucose (2g/l) | ++ |
| FVEhigh | + |
| Fucoidan Sigma | + |
| Carrageenan | +/- |
| Heparin | + |

**Enzymatic Degradation Experiments**

No enzyme could be produced from *Saccharophagus degradans* cultivations. Cell disruption solutions did show a non distinct ability to degrade FVElow (data not shown). Other polysaccharides were not degraded by neither cell disruption solution nor supernatant.

### 4.3.5 *Pseudoalteromonas atlantica* DSM 6839

*Pseudoalteromonas atlantica* was chosen because of its described ability to degrade fucoidan (Yaphe and Morgan, 1959). Figure 64 shows the growth curves for *Pseudoalteromonas atlantica* with three different C-sources. Here also only absorption is plotted. The consumption of FVEhigh was very weak.

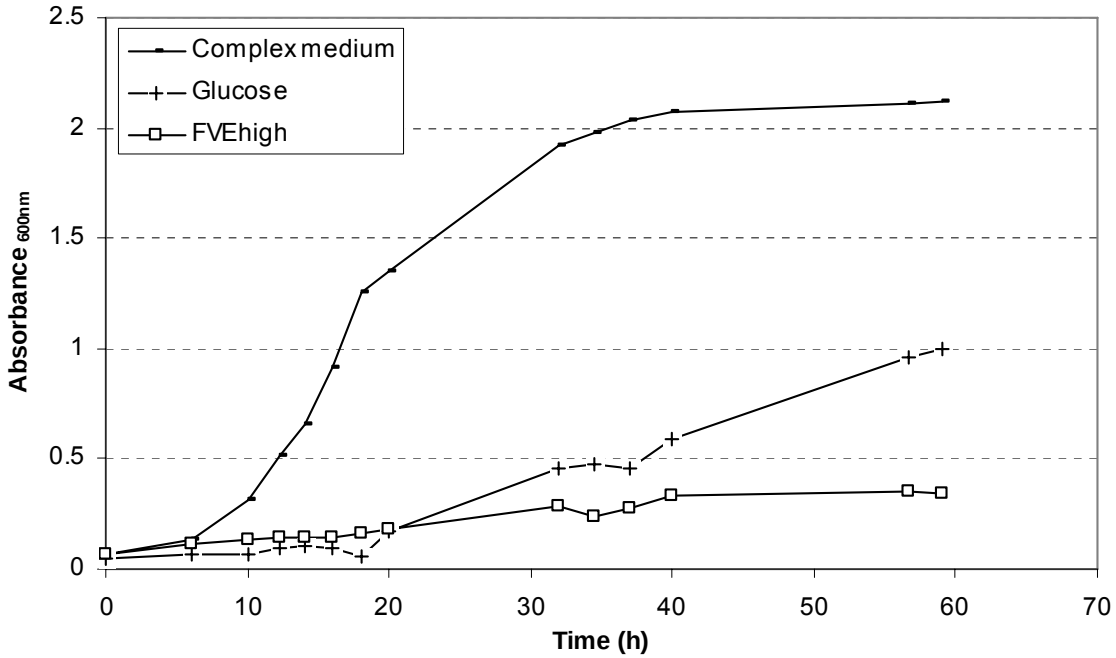



**Figure 64: *Pseudoalteromonas atlantica*: Growth curves on minimal medium with different C-Sources; 27 °C**

**Cultivation Experiments**

Table 17 shows the results of cultivations with *Pseudoalteromonas atlantica* on minimal medium with different C-sources. *Pseudoalteromonas atlantica* showed a slight utilisation of FVEhigh and Fucoidan Sigma. Compared to its ability to utilise glucose, this feature can be seen as a fucoidan-degrading ability.

**Table 17: *Pseudoalteromonas atlantica*: Cultivation experiments on minimal medium; +++ = very good growth; ++ = good growth; + = slight growth; - = no growth**

| C-source | growth |
|---|---|
| Complex medium | ++ |
| D-Glucose (2g/l) | + |
| FVEhigh | + |
| Fucoidan Sigma | + |

**Enzymatic Degradation Experiments**

No enzyme could be produced from *Pseudoalteromonas atlantica* cultivations. Cell-free supernatant did not show any enzyme activity on any of the sulphated polysaccharides (data not shown). There are hints that *Pseudoalteromonas atlantica* enzyme preparations from cell disruptions are able to degrade FVElow (data not shown). No cell debris were analysed for their degradation potential.

### 4.3.6 *Pseudoalteromonas carrageenovora* DSM 6820

*Pseudoalteromonas carrageenovora* was also chosen because of its described ability to degrade fucoidan (Yaphe and Morgan, 1959).

**Cultivation Experiments**

Figure 65 shows growth curves for the cultivation of *Pseudoalteromonas carrageenovora* on minimal medium with different C-sources. *Pseudoalteromonas carrageenovora* grew very well in complex media and with glucose as single C-source, but only showed slight consumption of FVEhigh. Due to biofilm formation this growths could not be detected satisfactorily by gravimetrical measurements, thus only the absorption is plotted.

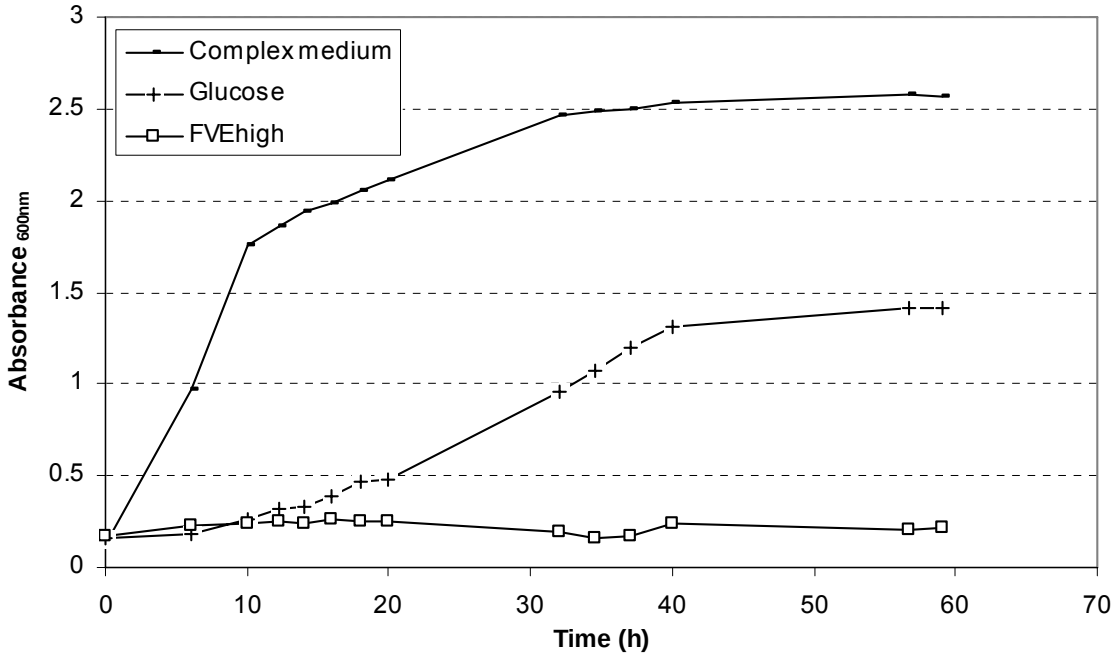



**Figure 65: *Pseudoalteromonas carrageenovora*: Growth curves on minimal medium with different C-sources**

Table 18 shows the results of cultivations with *Pseudoalteromonas carrageenovora* with different C-sources. *Pseudoalteromonas carrageenovora* was able to utilise heparin as single C-source and was slightly able to utilise FVEhigh, Fucoidan Sigma and carrageenan.

**Table 18: *Pseudoalteromonas carrageenovora*: Cultivation experiments on minimal medium; +++ = very good growth; ++ = good growth; + = slight growth; - = no growth**

| C-source | growth |
|---|---|
| Complex medium | ++ |
| D-Glucose (2g/l) | ++ |
| FVEhigh | + |
| Fucoidan Sigma | + |
| Carrageenan | + |
| Heparin | ++ |

### Enzymatic Degradation Experiments

No enzyme could be produced from *Pseudoalteromonas carrageenovora* cultivations. The supernatant of *Pseudoalteromonas carrageenovora* was only slightly active on laminarin (data not shown). Figure 66 shows the enzymatic degradation (absorbance) with cell disruption solutions of *Pseudoalteromonas carrageenovora*. It shows that *Pseudoalteromonas carrageenovora* cell disruption solutions were active on laminarin and

on FVElow, but were not on FVEhigh and Fucoidan Sigma. Cell debris were not tested for their degradation potential.

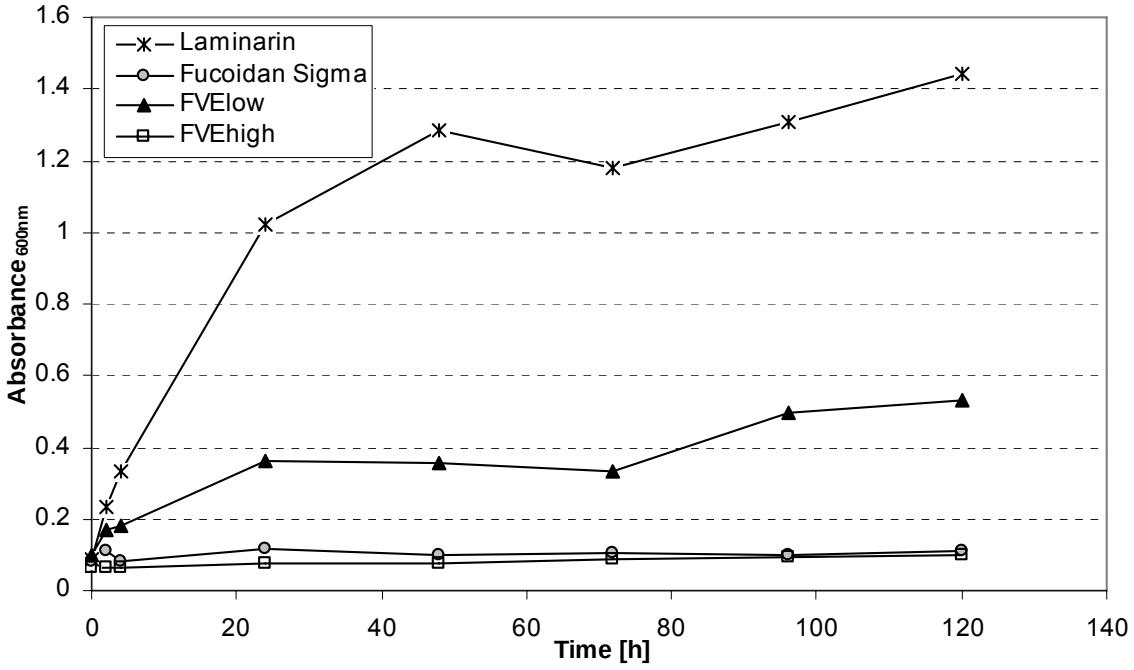



**Figure 66: *Pseudoalteromonas carrageenovora*: Enzymatic degradation with cell disruption solutions at 27 °C**

## 4.3.7 *Pedobacter heparinus* DSM 2366

*Pedobacter heparinus* was chosen because of its ability to degrade heparin. As the heparin molecule has certain similarities to the fucoidan molecule it was postulated that the same enzyme system could be used for the degradation of both polysaccharides. With the successful degradation of heparin in a whole cell conversion (cultivation experiment), it was possible to calibrate the newly set up SE-HPLC system. Figure 67 shows the whole cell conversion of heparin in association with biomass production.

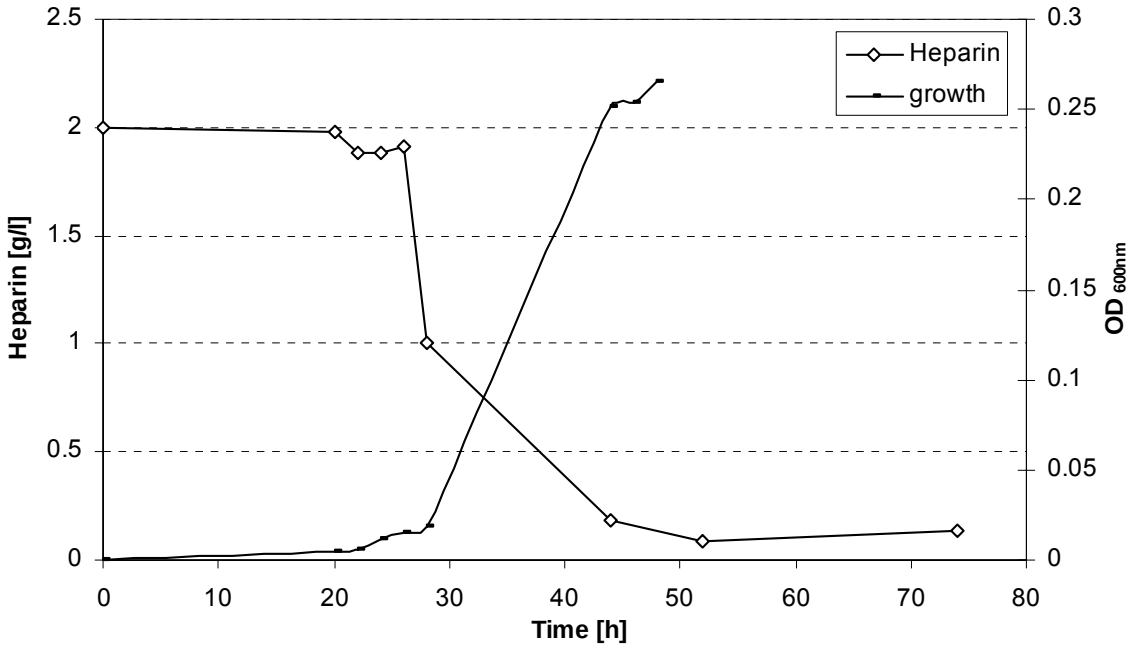



**Figure 67: Whole cell conversion of heparin (detected by SE-HPLC-analysis) during cultivation of *Pedobacter heparinus* DSM 2366 in a shake flask (1l) on minimal medium, 27 °C, 100 min$^{-1}$, supplemented with 2g/l heparin**

## Cultivation Experiments

Table 19 shows the tested whole cell consumption abilities of *Pedobacter heparinus*. However, only slight FVEhigh and Fucoidan Sigma consumption could be shown. As expected, *Pedobacter heparinus* was utilising heparin as single C-source (also stated in Figure 67).

**Table 19: *Pedobacter heparinus*: Cultivation experiments on minimal medium;**
**+++ = very good growth; ++ = good growth; + = slight growth; - = no growth**

| C-source | growth |
|---|---|
| Complex medium | ++ |
| D-Glucose (2g/l) | ++ |
| FVEhigh | + |
| Fucoidan Sigma | +/- |
| Carrageenan | ++ |
| Heparin | ++ |

**Enzymatic Degradation Experiments**

No pure enzyme could be isolated from *Pedobacter heparinus* cultivations. There are hints that *Pedobacter heparinus* was able to slightly degrade FVElow both with a supernatant solution and a cell disruption solution (data not shown).

**Problems arising during analysis – Auto hydrolysis (Chemical-Physical Hydrolysis):**

Enzymatic degradation was performed at 27 °C and 38 °C. At 27 °C in some cases no enzymatic degradation of the sulphated polysaccharides could be detected. At 38 °C every polysaccharide seemed to be degraded. Further analyses revealed, that a spontaneous hydrolysis of the sugars took place.

The auto hydrolysis was in the range of 0.1 to 0.3 g/l and thus in the same range as the detected enzymatic degradation. Enzymatic degradation can therefore not be ascertained at higher temperatures. Figure 68 shows an example of the auto hydrolysis of FVElow which was the highest chemical-physical degradation detected in this thesis.

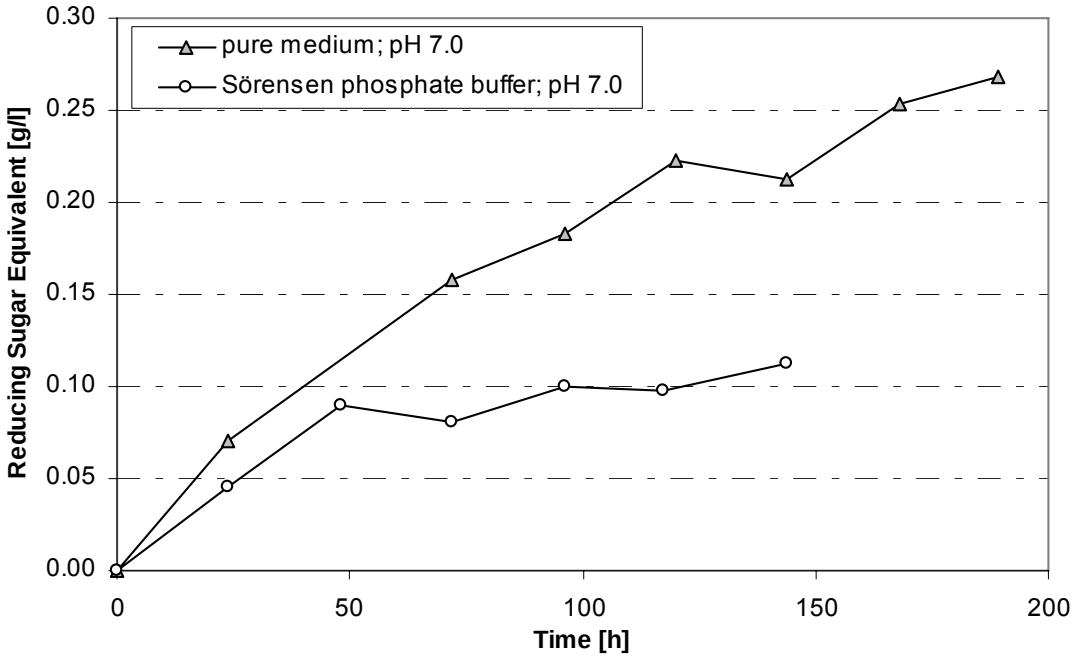



**Figure 68: Auto hydrolysis of FVElow at 38 °C**

### 4.3.8 Polysaccharide Degradation Tests with Commercially Available Enzymes

Several commercial enzymes were tested for their ability to degrade interesting (sulphated) heterogeneous polysaccharides. The results are presented in the following section.

**Laminarinase**

Laminarinase from *Trichoderma sp.* was tested on laminarin (0.004 g/ml) as optimal substrate as well as on FVEhigh (0.004 g/ml) and Fucoidan Sigma (0.004 g/ml). Figure 69 shows the result of the degradation of laminarin with 0.01 U/2.4 ml.

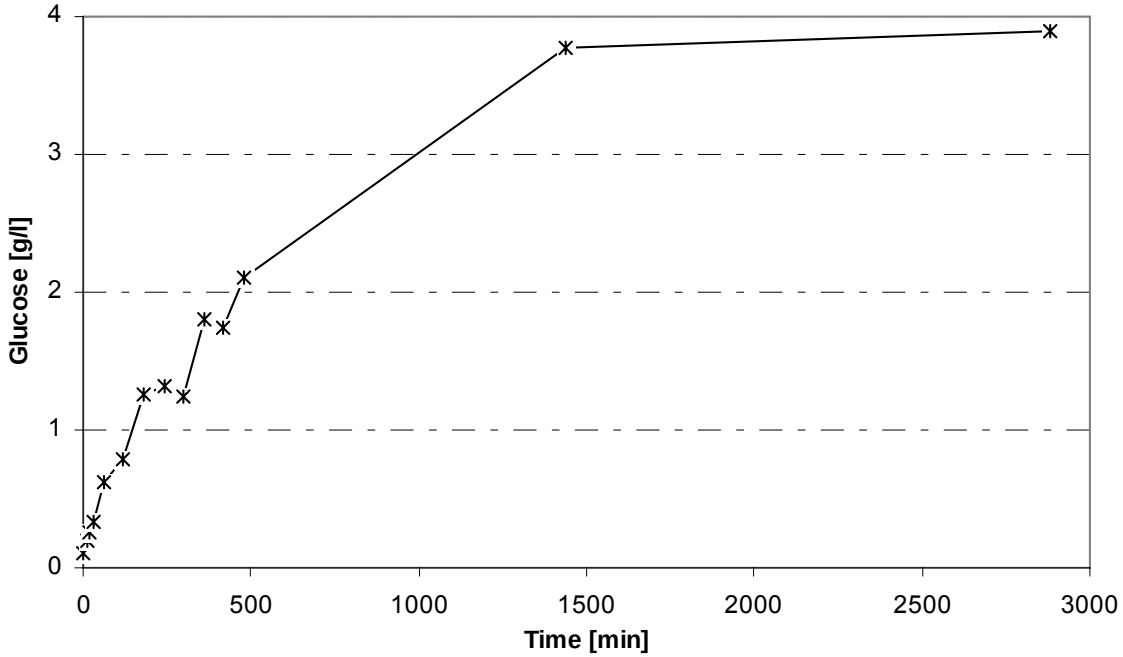



**Figure 69: Commercial enzyme test; laminarinase (0.01 U/2.4 ml) active on laminarin, releasing glucose-units, 37 °C, pH 5**

The degradation of laminarin could easily be followed as the reaction was not very fast. Conversion seemed to be complete at 1500 min as no more glucose units were released. As the enzymatic degradation of other substrates was much lower, these substrates are shown in Figure 70.

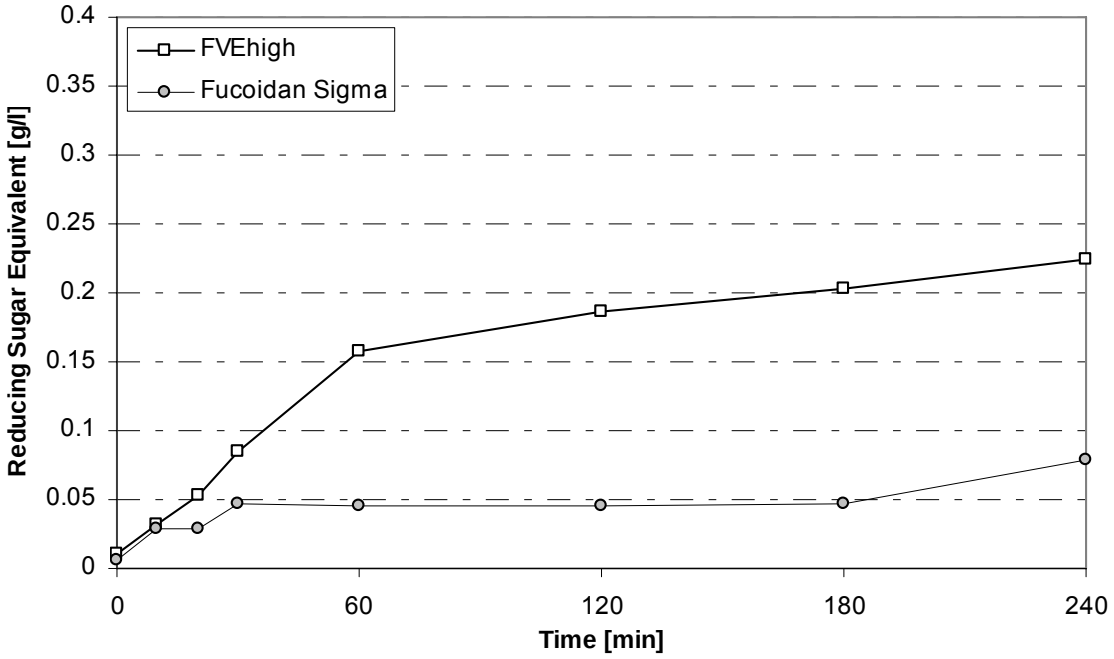



**Figure 70: Commercial enzyme test; laminarinase (0.01 U/2.4 ml) active on FVEhigh and Fucoidan Sigma; 37 °C, pH 5**

Laminarinase was slightly active on FVEhigh and even less active on Fucoidan Sigma.

### α -1→(3,4) Fucosidase from *Xanthomonas manihotis*

Figure 71 shows the results of the enzyme test performed on FVEhigh and Fucoidan Sigma. α-1→(3,4) Fucosidase from *Xanthomonas manihotis* was slightly active on FVEhigh and Fucoidan Sigma and was not active on laminarin and FVElow (data not shown).

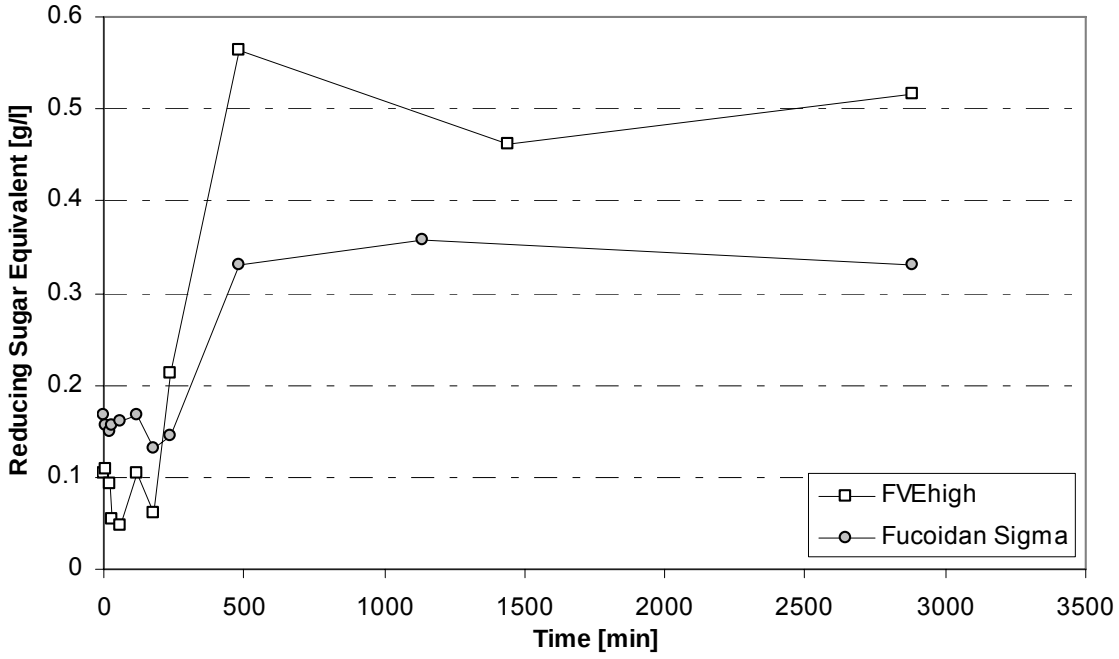



**Figure 71: Commercial enzyme test; α-1→(3,4) fucosidase (0.01 U/2.4 ml) active on FVEhigh and Fucoidan Sigma at 37 °C, pH 5**

### α-L-Fucosidase from bovine kidney

α-L-Fucosidase from bovine kidney was active on p-nitrophenyl-alpha-L-fucopyranoside. The data are shown in Figure 72. It was a very fast reaction, so only a few data points could be detected.

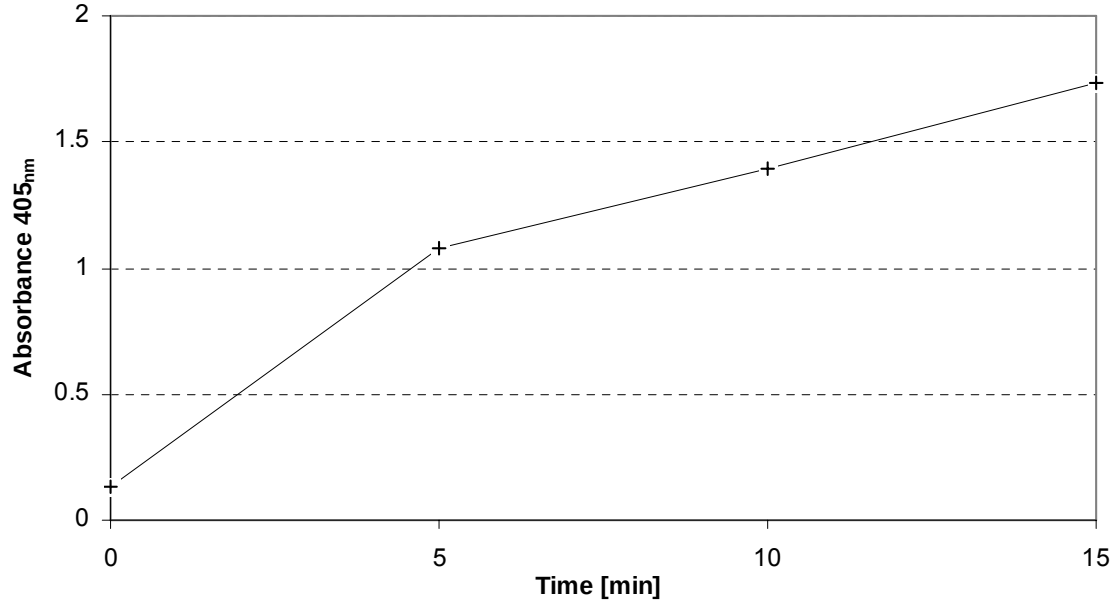



**Figure 72: Commercial enzyme test; α-L-fucosidase (0.5 U/0.033 ml) active on P-nitrophenyl-α-L-fucopyranoside at 37 °C, pH 6.0 building p-nitrophenol which can be measured directly**

P-nitrophenyl-α-L-fucopyranoside displayed a relatively slow rate of spontaneous degradation (data not shown). After 24h, an absorption of 0.4 was reached. No tests were performed on FVEhigh, FVElow, Fucoidan Sigma and LDEhigh until now, due to the high enzyme costs.

**α-Glucosidase**

As a negative control, α-Glucosidase was not active on p-nitrophenyl-α-L-fucopyranoside and p-nitrophenyl-sulphate (data not shown). Due to availability problems of the polysaccharides, it was not tested on FVEhigh, FVElow, LDEhigh, Sigma Fucoidan and laminarin.

### 4.3.9 Comparison of the Different Microorganisms

The following tables give a summary on the cultivation experiments as well as the enzymatic degradation experiments. Determination of growth was difficult due to biofilm formation, especially in the case of *Saccharophagus degradans*.

**Table 20: Influence of common carbon sources on the growth of selevted microorganisms. n.d. = not determined, (-) no growth, (+) slight growth, (++) good growth; (+++) very good growth**

| | Growth on | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | D-Glucose [g/l] | | | | | | | |
| Microorganism | Complex medium | 2 | 10 | 20 | 30 | D-Galactose | D-Xylose | D-Sucrose | D-Raffinose |
| *Dendryphiella arenaria* TM 94 | +++ | ++ | +++ | +++ | +++ | +++ | +++ | + | +++ |
| *Self Isolate WHV059* | +++ | ++ | +++ | +++ | +++ | ++ | ++ | ++ | +++ |
| *Self Isolate WHV012* | +++ | ++ | +++ | +++ | +++ | + | ++ | +++ | ++ |
| *Saccharophagus degradans* | ++ | ++ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| *Pseudoalteromonas atlantica* | ++ | + | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| *Pseudoalteromonas carrageenovora* | ++ | ++ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| *Pedobacter heparinus* | ++ | ++ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |

**Table 21: Influence of special polysaccharides on the growth of selected microorganisms. FVEhigh = self extracted fucoidan from *Fucus vesiculosus* high molecular weight; FVElow = self extracted fucoidan from *Fucus vesiculosus* low molecular weight**

| | Growth on | | | | | |
|---|---|---|---|---|---|---|
| **Microorganism** | **FVEhigh** | **Fucoidan Sigma** | **Carrageenan** | **Heparin** | **Dextran T2000** | **Starch** |
| *Dendryphiella arenaria* TM 94 | +/- | - | + | n.d. | + | +++ |
| *Self Isolate WHV059* | +/- | + | - | N .d. | +++ | + |
| *Self Isolate WHV012* | +/- | - | - | n.d. | - | ++ |
| *Saccharophagus degradans* | + | + | +/- | + | n.d. | n.d. |
| *Pseudoalteromonas atlantica* | + | + | n.d. | n.d. | n.d. | n.d. |
| *Pseudoalteromonas carrageenovora* | + | + | + | ++ | n.d. | n.d. |
| *Pedobacter heparinus* | + | +/- | ++ | ++ | n.d. | n.d. |

**Table 22: Enzymatic Degradation; FVEhigh = self extracted Fucoidan high molecular weight, FVElow = self extracted Fucoidan low molecular weight; n.d. = not determined, (-) negative, (+) slightly positive, (++) very positive, (+/-) not distinct**

| Strain/ Enzyme source and conditions | FVEhigh | FVElow | Fucoidan Sigma | Laminarin | Heparin |
|---|---|---|---|---|---|
| *Dendryphiella arenaria* TM 94 | | | | | |
| -supernatant; 27°C | - | - | - | ++ | n.d. |
| -supernatant; 37°/38°C | - | - | - | ++ | n.d. |
| -intra cellular proteins ; 27°C | n.d. | n.d. | n.d. | n.d. | n.d. |
| *Self Isolate WHV059* | | | | | |
| -supernatant; 27°C | n.d. | n.d. | n.d. | n.d. | n.d. |
| -supernatant; 37°/38°C | n.d. | n.d. | n.d. | n.d. | n.d. |
| -intra cellular proteins ; 27°C | - | - | - | ++ | n.d. |
| *Saccharophagus degradans* | | | | | |
| -supernatant; 27°C | n.d. | - | n.d. | n.d. | n.d. |
| -supernatant; 37°/38°C | - | +/- | - | - | n.d. |
| -intra cellular proteins ; 27°C | - | +/- | - | +/- | n.d. |
| *Pseudoalteromonas atlantica* | | | | | |
| -supernatant; 27°C | - | - | - | n.d. | n.d. |
| -supernatant; 37°/38°C | - | ++ | - | n.d. | n.d. |
| -intra cellular proteins ; 27°C | + | ++ | + | + | n.d. |
| *Pseudoalteromonas carrageenovora* | | | | | |
| -supernatant; 27°C | - | +/- | - | + | n.d. |
| -supernatant; 37°/38°C | - | + | - | + | n.d. |
| -intra cellular proteins ; 27°C | - | + | - | ++ | n.d. |
| *Pedobacter heparinus* | | | | | |
| -supernatant; 27°C | - | - | - | n.d. | - |
| -supernatant; 37°/38°C | - | + | - | n.d. | + |
| -intra cellular proteins ; 27°C | - | + | - | n.d. | ++ |

### 4.3.10 Summary - Microorganisms with a Fucoidan Degrading Potential

To determine the ability of different (marine) microorganisms to degrade fucoidan, several experiments were performed. Both fucoidan as single C-source was tested as well as induced cultivation with fucoidan as additional C-source. The ability to grow on fucoidan as single C-source indicated that the microorganism was able to degrade fucoidan.

The results were contradictory in some cases. Strains that seemed to be able to degrade Fucoidan Sigma and FVEhigh as a C-source were *Pseudoalteromonas atlantica* and *Pseudoalteromonas carrageenovora, Pedobacter heparinus* as well as *Saccharophagus degradans*. The results with FVEhigh were contradictory for *Dendryphiella arenaria* TM 94, Self Isolate WHV059 and Self Isolate WHV012. Self Isolate WHV059 was able to use Fucoidan Sigma as single C-source.

In a cell-free environment the best results were achieved with cell disruption solutions. Here, in addition to the bacteria, Self Isolate WHV059 seemed able to degrade FVElow. *Pseudoalteromonas atlantica* showed the best FVElow-degrading ability with a cell disruption solution. *Pseudoalteromonas carrageenovora* showed a very high intracellular (or membrane-bound) laminarin-degrading ability. Extra cellular enzyme activity was only detected for *Dendryphiella arenaria* TM 94 and *Pseudoalteromonas atlantica* against laminarin. The auto hydrolysis of FVElow at 38 °C hampered analyses.

## 4.4 Metabolites of Two Fungi with a Fucoidan-Degrading Potential

Cultivations of *Dendryphiella arenaria* TM 94 and Self Isolate WHV059 were performed under optimal conditions. Representative samples were taken from these cultivations and solvent extracts were produced from supernatants and cells after certain incubation periods. Methyl-*tert*.-butyl-ether (MTBE) was used for the extraction of the supernatants, methanol/chloroform (1:1) for the cells. Figure 73 shows the resulting organic solutions, which were analysed by thin layer chromatography or applied in anti-microbial tests, respectively.

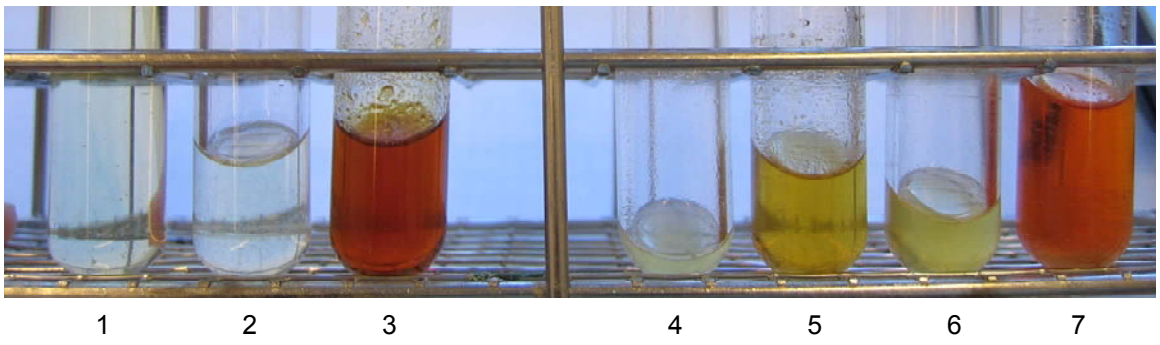



**Figure 73: Solvent extracts; 1 = MPY-medium; 2 = supernatant from *D. arenaria* TM 94 (4 days incubation); 3 = cells from *D. arenaria* TM 94 (4 days incubation); 4 = supernatant from *D. arenaria* TM 94 (7 days incubation); 5 = cells from *D. arenaria* TM 94 (7 days incubation); 6 = supernatant from Self Isolate WHV059 (7 days incubation); 7 = cells from Self Isolate WHV059 (7 days incubation) all dissolved in DMSO**

### 4.4.1 Analysis of Fungal Metabolites by Thin Layer Chromatography (TLC)

The produced extracts were applied on TLC silica gel plates 60 $F_{254}$ aluminium (Merck) for analysis. Development of the plates was performed in a development chamber using 4% methanol in chloroform. Visualisation was conducted through various spray reagents with succeeding incubation at 110 °C for 5-10 min. Anisaldehyde visualises polyols, steroids and terpenes, 5% sulfuric acid in methanol visualises organic substances.

Figure 74 shows extracts of *D. arenaria* TM 94 of the 4 days incubation experiments. The left part of the figure (A) shows the visualisation with anisaldehyde and the right part (B) the visualisation with 5% sulphuric acid in methanol. Compared to the original medium extract, especially the cell extract showed additional spots, both with higher and lower $R_F$-values (see arrows).

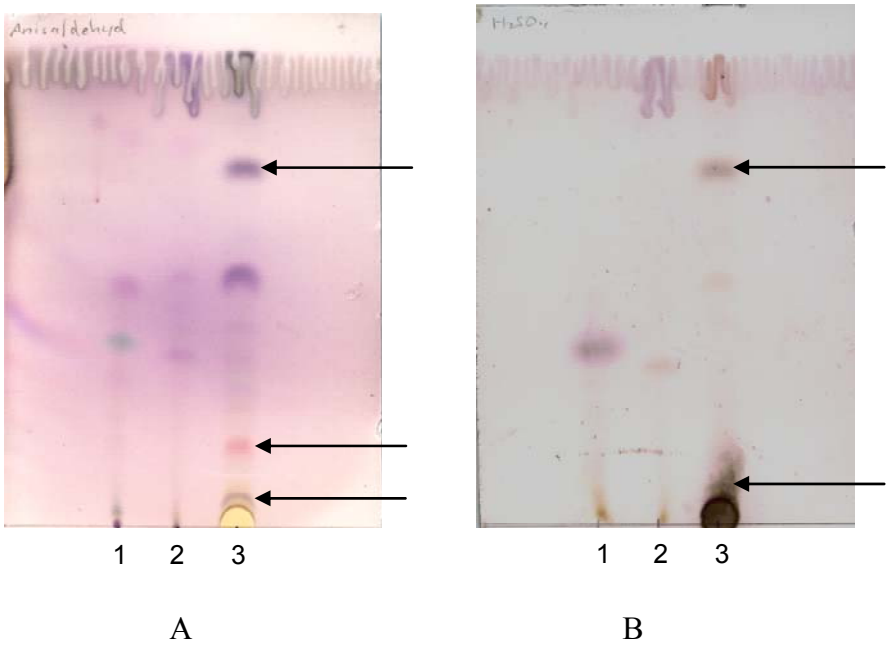



**Figure 74: Visualisation of the extracts (10 µl) of *Dendryphiella arenaria* TM 94 on TLC plates. A = visualisation by anisaldehyde; B = visualisation by 5% sulphuric acid in methanol; 1 = MPY medium in MTBE; 2 = supernatant ( 4 days incubation) in MTBE; 3 = cell extract ( 4 days incubation) in methanol/chloroform**

Figure 75 shows the visualisation of extracts from *Dendryphiella arenaria* TM 94 and Self Isolate WHV059 after 7 days of incubation. Visualisation was also achieved by anisaldehyde (A) and 5% sulphuric acid in methanol (B).

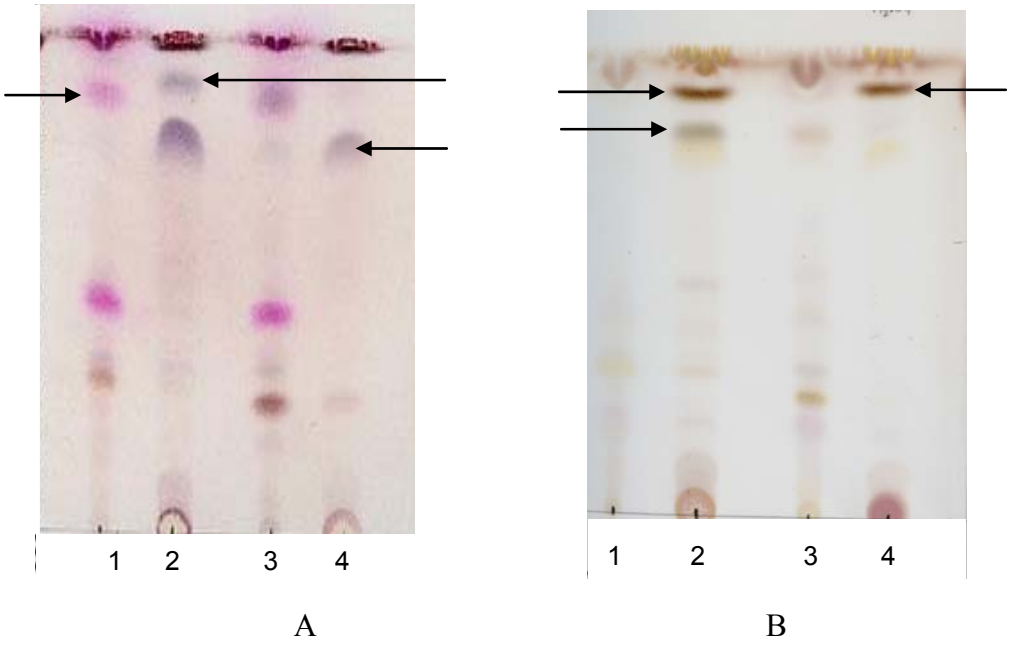



**Figure 75: Visualisation of the extracts (10 µl) of *Dendryphiella arenaria* TM 94 and Self Isolate WHV059 (after 7 days of incubation) on TLC plates. Solvent system: 4% $CH_3OH$ in $CHCl_3$ A = visualisation by anisaldehyde; B = visualisation by 5% sulphuric acid in methanol; 1 = supernatant (*D. arenaria*) in MTBE; 2 = cell extract (*D. arenaria* TM 94) 3 = supernatant (Self Isolate WHV059) in MTBE; 4 = cell extract (Self Isolate WHV059)**

### 4.4.2 Tests for Anti-microbial Activity

After evaporation of the original solvents (MTBE, CHCOH:$CHCl_3$), and resolution in dimethylsulfoxide (DMSO), the extracts from *Dendryphiella arenaria* TM 94 and Self Isolate WHV059 cultivations were analysed against the fungi *Microbotryum violaceum* and *Saccharomyces cerevisiae* as well as against the bacteria *Bacillus subtilis* and *Escherichia coli*. The fungi were incubated for 3 to 4 days on yeast-peptone-dextrose (YPD) agar plates at room temperature, *Bacillus subtilis* was incubated on meat-extract-peptone (MEP) agar and *Escherichia coli* was incubated on Luria-Bertani (LB) medium, all at 27 °C. For comparison well-known antibiotics were used in additional tests. Zones of inhibition were measured. Inhibition strengths compared to the diameter of the zone of inhibition are shown in Table 23.

**Table 23: Inhibition strengths referred to the diameter of the zone of inhibition**

| Diameter of zone of inhibition | Inhibition strengths |
|---|---|
| 0 mm | no inhibition |
| < 20 mm | weak inhibition |
| 20 – 30 mm | average inhibition |
| >30 mm | strong inhibition |

Amphotericin B showed zones of inhibition against *M. violaceum* of 19-21 mm and against *S.cerevisisae* of 19-22 mm in diameter. Penicillin G was active against *B. subtilis* (20-22 mm) and Chloramphenicol was a potent suppressor of *E. coli* (36 mm). The extracts of *Dendryphiella arenaria* TM 94 and Self Isolate WHV059 did not show any anti-fungal and anti-bacterial activity compared to antibiotics (no zone of inhibition was performed).

# 5 Discussion

The discussion of the results of this thesis is divided into three major parts to enable the independent review of the function of fucoidan in medical applications or as a substrate for microorganisms. The first part deals with the extraction of the sulphated polysaccharides and their characterisation and modification. The second part examines the bioactivity of these polysaccharides and its potential application e. g. in medicine. The third and last part deals with the microorganisms with a potential fucoidan-degrading ability.

## 5.1 Sulphated Polysaccharides/Oligosaccharides

One big point of the project was to produce a standard fucoidan out of the heterogeneous polysaccharides, which was performed successfully. Problems arose through the very low yield of the existing methods and the varying structure of the fucoidans due to seasonal variations (Black, 1954). Therefore, the extraction procedure had to be monitored very carefully and the extracts produced had to be checked for their quality quite often.

The second step was to establish different methods to evaluate the quality of the fucoidan produced at the institute without needing to send away the samples and to be forced to wait for the results. This helped to speed up the production process. During this project several different methods were tested and developed for the special application of fucoidan analysis.

When fucoidan was produced from *Fucus vesiculosus* only quite small amounts could be extracted. The yield was around 1% in all experiments performed and could not be improved significantly. The quality of the extract, however, was constant. The extraction procedure led to different kinds of fucoidan, whereof the biggest fraction was around 1,300 kDa with a relatively high sulphatation grade of 8.45 %. Compared to literature values this is a relatively high, although not implausible, molecular weight. Fractionation of the extracts led to a second smaller fraction of around 30-50 kDa with a somewhat lower sulphatation degree. The yield of these smaller polysaccharides was even lower than for the bigger ones, since the extraction procedure was aimed at the bigger molecules. Through fractionation and dialysation fucoidan could be successfully purified and a high product quality could be achieved. A comparison of the constituents of our fucoidans with the literature is shown in Figure 76.

Fucoidans isolated from *Laminaria digitata* (LDEhigh) showed slightly different properties even though they were extracted by the same procedure. This indicates the differences between the fucoidans of the two algae.

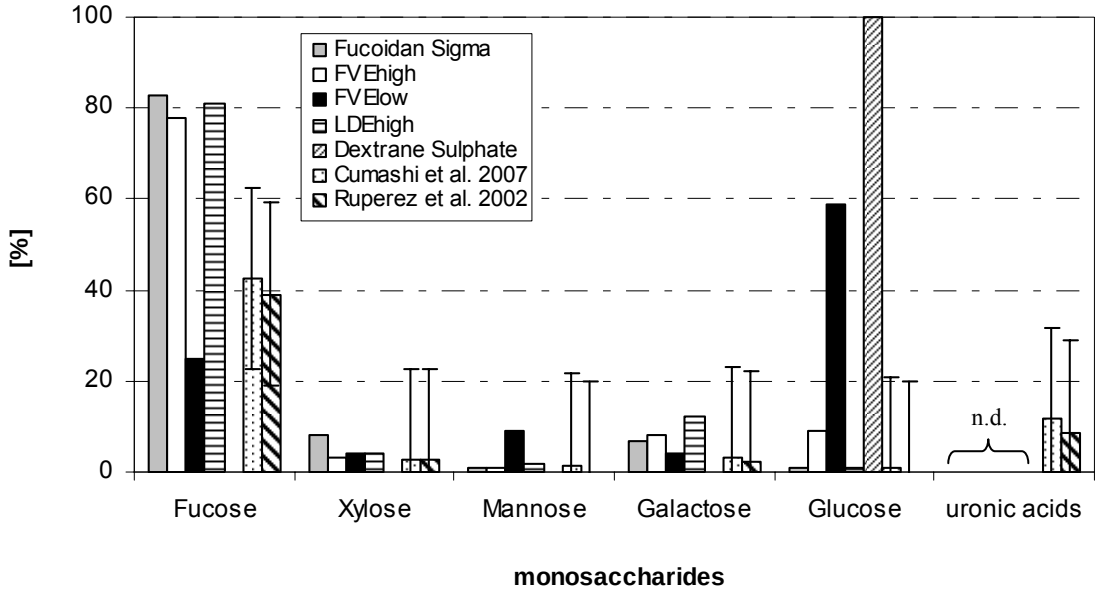



**Figure 76: Monosaccharide composition of different polysaccharides**

At the time that this project was initiated, it was not possible to estimate the molecular mass of oligosaccharides due to the absence of appropriate standards (Pomin et al., 2005), and it was necessary to establish an analysis system. With the developed large C-PAGE-system it was possible to monitor sulphated polysaccharides before and after degradation and to see the creation of smaller oligosaccharides. It was not possible to excise these oligosaccharides from the gel and to analyse them further due to the low extraction yield.

The use of elemental analysis for further structure elucidations worked very well and the amount of sulphate groups could be evaluated. Elemental analysis can also be used as a quality insurance, whether the sulphate groups are still with the molecule or if they have been lost in purification steps.

Modification experiments with FVEhigh, FVElow, Fucoidan Sigma and LDEhigh were only possible with chemical degradation. No physical degradation could be detected, likely because the impact on the sugar via ultrasound was too weak or because the given detection methods were insufficient to monitor degradation. The latter reason is more likely, since our cooperation partner has previously reported ultrasonic degradation of these molecules. With chemical degradation, however, the production of oligosaccharides

is possible. The degradation can be monitored with colorimetric methods, as well as with the C-PAGE system and SE-HPLC-system. Enzymatic degradation is discussed in chapter 5.3.

One big goal that could be achieved during this thesis was the production and characterisation of fucoidan from *Fucus vesiculosus*. It would be very interesting to produce even more fucoidan to be able to produce more oligosaccharides. A structural proposal could be given based on the data collected throughout this thesis.

## 5.2 Bioactivity

To determine potent bioactivities in the produced fucoidan gives an opportunity to describe the potential application of the given products. Luckily different bioactivities could be tested. In literature there are several activities that are said to be possessed by fucoidan. To evaluate the bioactive potential of fucoidan after performing all these bioactivity tests it is obvious that the given fucoidan from *Fucus vesiculosus* shows a very high potential for further usage.

**Anti-coagulant Activity**

The most commonly described bioactivity of fucoidan is the anti-coagulant activity. To put our results into perspective, some examples of fucoidan extracted from *Fucus vesiculosus* are given in the following paragraph. The first detailed study of blood anti-coagulant polysaccharides from *Fucus vesiculosus* was published by Springer et al., who showed that the measured anti-coagulant activity exceeded that of heparin (Shanmugam and Mody, 2000; Springer et al., 1957). Due to these findings several groups have since tested fucoidan from *Fucus vesiculosus* for anti-coagulant activity.

In 1957 Adams and Thorpe (Shanmugam and Mody, 2000) described that the activity of fucoidan was between 8.9 and 9 heparin units/mg. Church described an anti-coagulant action in 1989 (Church et al., 1989). The sulphation degree seemed to be closely connected to the bioactivity (Grauffel et al., 1989; Qiu et al., 2006). The fucans with a high sulphate content present a significantly increased anti-coagulant activity (Grauffel et al., 1989). The size of the molecules might also be of interest: 'The relatively low molecular-mass fractions (50 kDa) retained high anti-coagulant activities' (Grauffel et al., 1989); 'the anti thrombin activity was found to be dependant on the molecular weight (50 kDa)' (Nishino et al., 1991). These results could not be confirmed by our own measurements. It has to be taken into consideration that our smaller molecules are not fucoidans in the lower sense and can as such not be compared with results concerning low molecular weight fucoidans. To produce low molecular weight fucoidans, the FVEhigh has to be enzymatically or chemically degraded.

In 2000 Thorlacius found that fucoidan dramatically delayed the progression of thrombus growth and the time required for complete vessel occlusion (Thorlacius et al., 2000). This is supported by Mauray who showed that fucoidan treatment greatly prolongs the prothrombin time (decreased Quick percentage) (Mauray et al., 1995; Thorlacius et al.,

2000). Fucoidan treatment is greatly prolonging the prothrombin time (decreased Quick percentage) (Thorlacius et al., 2000). These results correlate with our findings. However, they cannot be described as 'greatly prolonging', since only a slight increase in the Quick percentage could be measured. It would be very interesting to repeat these analyses with human blood and more measurements. Differences in the mechanism of the anti-coagulancy may lie in the structure of the sulphated polysaccharides (Pereira et al., 1999), or even in the steric conformation related to the sugar components and the positions of the glycosidic linkage and the sulphate group (Nishino et al., 1991).

However, literature on the mechanism of action of fucoidan is complex and partly contradictory (Thorlacius et al., 2000): In 2007 Cumashi published a comparative study of the anti-inflammatory, anti-coagulant, anti-angiogenic, and anti-adhesive activities of nine different fucoidans from brown seaweeds (Cumashi et al., 2007). In these analyses the fucoidan isolated from *Fucus vesiculosus* could not prevent thrombin-induced platelet aggregation in agreement with Springer (Shanmugam and Mody, 2000; Springer et al., 1957), but showed that its interaction with the heparin cofactor II is enormous. As the structure of the FVEhigh and FVElow is not fully elucidated yet, it is very difficult to determine which mechanism lies behind the detected anti-coagulant activity. It would be very interesting to further investigate the structure/function relation. Different anti-coagulant tests aimed at different factors could be analysed with our fucoidan in order to further elucidate the mechanism. Unfortunately it was not possible to investigate the anti-coagulant activity of FVEhigh, FVElow and LDEhigh on human blood samples due to safety regulations.

**Anti-viral Activity**

Anti-viral activity of sulphated polysaccharides was first described some time ago. In 1987 Gonzalez et al. described the anti-viral activity of carrageenan against several viruses (Gonzalez et al., 1987). They suggested that anti-virally inert polysaccharides are activated by the presence of sulphate groups. In our experiments against HCMV, laminarin, a non-sulphated polysaccharide was used as a negative control. There are several publications concerning the anti-viral bioactivity of fucoidan isolated from *Fucus vesiculosus* that are of interest for our results:

In 1988 Baba (Baba et al., 1988) described the inhibition of various enveloped viruses including Herpes simplex virus. In 1993 Beress et al. (Beress et al., 1993) described an anti-HIV activity of some fractions of self produced *Fucus vesiculosus* fucoidan. The $IC_{50}$

value shows the concentration at which the virus load is 50% of the starting value, and a small $IC_{50}$ value is thus linked to a good virus repellent. In our tests, three of the produced fucoidans showed an anti-viral activity. The activity of FVEhigh and Fucoidan Sigma, however, exceeded the activity of FVElow. Laminarin could be successfully adapted as a negative control. FVEhigh shows a very good antiviral activity and tests would thus be rewarding. Compared to commercial antivirus drugs, e.g. ganciclovir ($IC_{50}$ = 14µg/ml; Figure 23), FVEhigh is a very promising antivirus drug. Tests were performed in order to show the influence of FVEhigh against the host cell as well. FVEhigh did not show any cytotoxic effects. Higher concentrations of FVEhigh are not likely in the application and were thus not tested. This means FVEhigh is a very promising antivirus drug with no harmful influence on the host cell. Fucoidan could thus be an alternative anti herpetic drug. Fucoidans from other algae than *Fucus vesiculosus* also show high activities against various viruses. Some fractions of the brown seaweed *Adenocystis utricularis* with a high fucose content display very small $IC_{50}$ values against HSV-1 and 2 (Ponce et al., 2003). Herpes simplex Virus is also successfully inhibited by fucoidan from *Undaria pinnatifida* (Hemmingson et al., 2006; Lee et al., 2004a). Another thoroughly investigated polysaccharide for anti-viral bioactivity is carrageenan (Carlucci et al., 1997a; F-Tischer et al., 2006; Gonzalez et al., 1987). Unfortunately no self-extracted carrageenan was produced in this thesis that could be compared to literature values.

**Anti-tumour Activity**

The anti-tumour activity of fucoidan from *Fucus vesiculosus* (Sigma Fucoidan) has been tested previously by Aisa et al. (Aisa et al., 2005). It was found that the fucoidan inhibited proliferation and induced apoptosis in human lymphoma HS-sultan cell lines. It was indicated that fucoidan is inducing apoptosis through a mitochondrial pathway. Other sulphated polysaccharides also show anti-tumour activity such as carrageenan (Yuan and Song, 2005). Our experiments are in the early beginning, however the anti-tumour tests performed in Japan show quite promising results. As expected, the Fucoidan Sigma showed a high anti-tumour activity. The self-extracted sulphated polysaccharides FVEhigh, FVElow and LDEhigh also showed an anti-tumour activity. It would be very interesting to analyse how the anti-tumour activity is influenced by the molecular size of the self-extracted molecules. This could be achieved by applying the FVEs and LDEhigh against the mouse skin cancer and Epstein Barr Virus early antigen promotion again, after enzymatic or chemical degradation.

**Antibodies**

The detection of an antibody against fucoidan gives the opportunity to monitor fucoidan dosages in, for example, patients, leading to a potent bio detection tool. The data collected for our new fucoidan antibody so far is not sufficient to produce such a tool, but the results are very encouraging. In 1990 Eardley et al. (Eardley et al., 1990) produced an antibody against sulphated polysaccharides. Ten years later Nakagawa et al. patented an antibody against fucoidan from *Kjellmaniella crassifolia* (Nakagawa et al., 2000) and in 2005 an antibody against fucoidan of *Undaria* was successfully used to detect fucoidan in human plasma after oral ingestion (Irhimeh et al., 2005). It would be interesting to analyse all our antibody libraries for potent fucoidan binders. All self isolated extracts should be analysed potentially leading to antibodies against FVEhigh, FVElow, Fucoidan Sigma (which has already been detected) and LDEhigh.

**Summary**

In this project the bioactivity against HCMV seems to be the most suitable application for FVEhigh and Fucoidan Sigma. As the self-extracted fucoidan FVEhigh has an $IC_{50}$ value of approximately 4 µg/ml, compared to the commercial virus static ganciclovir with a $IC_{50}$ value of 14 µg/ml (Figure 23), fucoidan seems to be a very potent virus static. One has to take into account that it is very important to ensure to produce fucoidan of the same quality every time. This might turn out to be very difficult as the composition suffers from seasonal variations (Black, 1954; Honya et al., 1999). For anti-tumour analysis the results are also very encouraging. FVEhigh and Fucoidan Sigma show anti tumour activity against TPA-induced skin cancer. FVElow and LDEhigh show a slightly weaker activity.

Anti-coagulant tests revealed, that the high molecular weight fucoidans are able to prolong the blood clotting time. A licensed project partner will be necessary for further experiments. An antibody against Fucoidan Sigma could be found. Further experiments with the other extracts could lead to even more antibodies. Research in this field is in the early beginning and can thus be perpetuated. The reason why fucoidan shows such a high variety of potential applications cannot be answered satisfactorily. Possibilities may lay in the different structural regions of the fucoidan with and without sulphation, possible branching sites and the size distribution within the extracted molecules. Intense analysis in this field may help to elucidate even more bioactivities.

## 5.3 Microorganisms with a Fucoidan-Degrading Potential

***Dendryphiella arenaria* TM 94**

The cultivation of *Dendryphiella arenaria* TM 94 did not give the previously expected results. The fungus did not express the described fucoidanase activity with the reported cultivation parameters. Two other *Dendryphiella* strains were supplied by Thomas de la Cruz (Institute of Microbiology, TU Braunschweig) for comparison with TM 94 and did not show any fucoidan degrading activity either. There are several possible explanations as to why the strain does not show any activity. For example, its ability might have been interpreted the wrong way. The first cultivations by Wu (Wu et al., 2002) were performed on solid state media that contained relatively high amounts of mono sugars that could be detected by the colorimetric methods used. Thus false results might have been reported. Another alternative is that the strain might have been shelved for too long; the project started in 2005 whereas the strain had lastly been used in 2002. There is the possibility that the fungus might have lost its ability to degrade fucoidan during storage. As the other *Dendryphiella* strains did not show any activity either, this possibility does not seem very likely. Bioreactor cultivations with *Dendryphiella arenaria* TM 94 showed that the fungus was still cultivable with very high yields in biomass. Even though the achieved biomass values of around 18 g/l with 10g/l supplemented glucose, were not very likely (usually 50% of glucose can be converted into biomass). This might be due to precipitation reactions during biomass measurements resulting in too high gravimetric values.
However, the decision was made during the project to look for other strains (commercially available as well as self-isolated) in order to find new fucoidanase producers. Until today no fucoidan-degrading fungus was published in literature.

**Self Isolate WHV059**

WHV059 was isolated in April 2005 and preliminarily named *Acremonium sp*., with no genetic screening performed. As this strain was not commercially available no optimal cultivation conditions could be applied. Several experiments were performed in order to optimise fucoidan-degrading ability of the strains. It proved able to degrade several different polysaccharides and should thus possess a polysaccharide degrading system. The results concerning fucoidan-degrading ability were not distinct.

**Self Isolate WHV012**

WHV012 was isolated in April 2005 and preliminarily named *Alternaria*. Not many experiments were performed with self Isolate WHV012 as it turned out that it might just be an ordinary ubiquitous fungus. Self Isolate WHV012 showed no distinct fucoidan-degrading ability against any of the given fucoidans.

***Saccharophagus degradans* DSM 17024**

*Saccharophagus degradans* seemed to be the strain with the highest potential to possess a fucoidan degrading system as several studies have shown its ability to degrade a large number of polysaccharides (Ekborg et al., 2005; Ekborg et al., 2006; Gonzalez and Weiner, 2000; Howard et al., 2004; Howard et al., 2003; Taylor Larry et al., 2006). A fucoidan-degrading ability has only been reported once, however. The experiments during this project showed that *Saccharophagus degradans* was able to degrade fucoidan, but the cultivation conditions may have to be optimised here as well.

Literature studies on *Saccharophagus degradans* revealed that its genomic sequence was fully elucidated in 2006. Its genome consists of 5057531 nucleotides including 4008 protein genes and 50 RNA genes. A fucoidanase could not be detected through the genomic sequence (KEGG), although Gonzalez (Gonzalez and Weiner, 2000) reports that *Saccharophagus degradans* is able to degrade fucoidan. The maximum enzyme activity has been detected in the logarithmic/stationary phase transition (Ekborg et al., 2005).

***Pseudoalteromonas atlantica* DSM 6839**

*Pseudoalteromonas atlantica* DSM 6839 showed a slight FVEhigh-degrading ability. It is well known in literature that microorganisms degrading fucoidan are often of the genus *Pseudoalteromonas* (Bakunina et al., 2002; Bakunina et al., 2000; Ivanova et al., 2002; Kusaikin et al., 2004; Perepolov et al., 2005; Yaphe and Morgan, 1959). Best results could be achieved with cell disruption solutions. This indicates, that *Pseudoalteromonas atlantica* DSM 6839 possesses an intra-cellular or membrane-bound enzyme with a fucoidan-degrading ability. Further experiments should be performed as well.

***Pseudoalteromonas carrageenovora* DSM 6820**

*Pseudoalteromonas carrageenovora* DSM 6820 was chosen, since it is able to degrade fucoidan (Yaphe and Morgan, 1959). Predominantly, it is able to degrade carrageenan (Guibet et al., 2007; Knutsen and Grasdalen, 1992). Carragenan is a very interesting

marine polysaccharide with similarities to fucoidan. In this thesis *Pseudoalteromonas carrageenovora* DSM 6820 showed contradictory results concerning the fucoidan-degrading ability. Best results could be achieved with cell disruption solutions on FVElow and laminarin. This indicates, that *Pseudoalteromonas carrageenovora* DSM 6820 possesses an intra-cellular or membrane-bound enzyme with a fucoidan-degrading ability. This is clearly a topic for future investigations.

***Pedobacter heparinus* DSM 2366**

*Pedobacter heparinus* was chosen, since this strain is able to degrade heparin. This was demonstrated of several different research groups (Shaya et al., 2006; Steyn et al., 1998). *Pedobacter heparinus* is the strain with the best assured ability to degrade this sulphated polysaccharide. As the detection system was not calibrated optimally at the start of the thesis, the degradation of heparin could be considered as the model degradation profile and was used to successfully calibrate the SE-HPLC system (see chapter 4.3.7).

On the other hand there was a big chance that *Pedobacter heparinus* could be able to degrade fucoidan as well, as it is also a sulphated polysaccharide. *Pedobacter heparinus* showed a slight fucoidan degrading ability. This might be due to the differences in structure between these two polysaccharides. Further analysis with this strain would be interesting.

**Commercial enzyme tests:**

Several commercially available enzymes were tested with their optimal substrates as well as with the different fucoidans.

*Laminarinase*

Laminarinase from *Trichoderma sp*. showed a very good degrading ability on laminarin, as expected. Experiments with FVEhigh revealed that laminarinase is also able to cleave this polysaccharide. This indicates, that FVEhigh is sharing structural characteristics with laminarin. Fucoidan Sigma is only slightly degraded, which indicates that the structure of Fucoidan Sigma differs from the one of FVEhigh. These differences may cause – as already stated – in the seasonal variations of fucose and the point of collection (Black, 1954; Honya et al., 1999). As the extraction procedure of the commercially available fucoidan is not known exactly, differences in the procedure may also alter the structure. Size analysis (see chapter 4.1.7) revealed, that Fucoidan Sigma was much smaller than

FVEhigh. If the recognised structure elements are of a bigger size, this may explain the lower degradation activity with Fucoidan Sigma.

*α-1→3,4 Fucosidase*

α-1→3,4 Fucosidase from *Xanthomonas manihotis* exhibited only a slight degrading ability on FVEhigh and even less with Fucoidan Sigma. Laminarin was not degraded at all, nor was FVElow. As α-1→3,4 Fucosidase releases non-reducing, terminal α-1,3-fucose and α-1,4 fucose from carbohydrates, which indicates that FVEhigh contains both of these glycosidic bonds. The problem with this enzyme is that it also shows other activities such as β-galactosidase-, α-mannosidase-, β-hexosaminidase-, neuraminidase-, α-1,6-fucosidase- and protease-activity. The detected degrading ability on FVEhigh and Fucoidan Sigma was very low, and may thus be one of the stated side reactions. It would be interesting to detect the α-1→3,4 Fucosidase activity on 4-methylumbelliferyl glycoside, as a unit is defined for this substrate. α-1→3,4 Fucosidase from *Xanthomonas manihotis* is a very interesting enzyme for our applications and should be analysed with other buffer conditions as well. Due to its high price, these experiments had been postponed.

*α-L-Fucosidase*

α-L-Fucosidase from bovine kidney was tested on its optimal substrate; p-nitrophenyl-α-L-fucopyranoside. It is a very fast reaction and could be analysed very well. Additional tests revealed that p-nitrophenyl-α-L-fucopyranoside is spontaneously degraded after 24h, the substrate solution can thus not be kept for very long and has to be freshly prepared each time. α-L-Fucosidase was also tested on potassium-4-nitrophenylsulphate as a negative control and showed no effect. It would be of great interest to test this enzyme also on FVEhigh, FVElow, Fucoidan Sigma and LDEhigh. These experiments also had been postponed because α-L-Fucosidase from bovine kidney was quite expensive.

*α-Glucosidase*

As expected, α-Glucosidase is not active on p-nitrophenyl-α-L-fucopyranoside and p-nitrophenyl-sulphate (data not shown). It was not tested on FVEhigh, FVElow, LDEhigh, Sigma Fucoidan and laminarin. These experiments could elucidate whether glucose-glucose-linkages are present in the polysaccharides.

For structure elucidations it would be very interesting to even use other enzymes to act on FVEhigh, FVElow, Fucoidan Sigma and LDEhigh.

For the α-linkages, besides the already named enzymes, α-amylase from *A.oryzae* as well as α-amylase from *B.licheniformis* would be interesting. These enzymes cleave the α-1,4 glycosidic bond in amylose. Pullulanase is cleaving α-1,4-glycosidic bonds from maltotriose units and α-L-iduronidase cleaves L-iduronate from dermatan sulphate and heparan sulphate. Another interesting enzyme complex is naringinase possessing a α-L-rhamnosidase activity which catalyses the cleavage of the linkage between terminal rhamnose and a glycone of rhamnose-containing glycosides and a *β*-D-glucosidase activity (EC 3.2.1.40) (Rau et al., 2001).

To further elucidate whether the self-extracted polysaccharides contain β-linkages, for example, cellulase from *Trichoderma reesei* could be interesting as well as other β-glucanases, for example from *S.cerevisiae* (Abd-El-Al and Phaff, 1968). Xylanases, for example an endo 1,4-β-xylanase from *Thermomyces lanuginosus* produced by submerged fermentation of a genetically modified *Aspergillus oryzae* microorganism degrade xylose containing polysaccharides and could give information whether a xylose-xylose-linkage is present. With sulphatases one could gain information about the sulphatation degree in connection to the sulphatation pattern. The list of enzymes for complex carbohydrate analysis is long and with the help of these enzymes the structure of the self-extracted polysaccharides might be revealed very well.

For example, laminarinase enzymes showed minimal activity on substrates with similar glucosidic bonds to those of laminarin, but different sizes and secondary and/or tertiary structures. The characteristics found in these enzyme systems may help to elucidate factors hampering rapid carbohydrate degradation. by prokaryotes (Alderkamp et al., 2007).

**Fungal Metabolites**

It is remarkable that no inhibition of *Microbotryum violaceum* was performed by *Dendryphiella arenaria* TM 94 extracts as this has been described in literature (de la Cruz, 2006). A possible reason for this lack of inhibition may be caused by the different cultivation procedure. De la Cruz did not cultivate the *Dendryphiella* in liquid culture as performed in this thesis, but on agar plates. Morphology of the fungus is different on agar plates and liquid culture, thus a different production of fungal metabolites, can be assumed. As a rule, the amount of metabolites produced on agar plates is higher than in liquid culture (B. Schulz, Institute of Microbiology (personal communication). The incubation

period might also have been too short. As the TLC analysis shows, different metabolites are built at different times. A longer incubation may evoke other metabolites. Therefore, investigations are still interesting to develop as marine microorganisms are a potent source for highly bioactive compounds (Biabani and Laatsch, 1998) such as anti-oxidant (Abdel-Lateff et al., 2002) or anti-tumoral (Lang et al., 2004; Langer et al., 2006).

**Summary for Microorganisms with a Fucoidan-Degrading Ability**

The production of fucoidan-degrading enzymes is still in its infancy. In recent years several research groups have been able to isolate potent bacteria and to degrade fucoidan. Only a few have been able to isolate the corresponding enzyme and to elucidate its structure. No group except Wu et al. (Wu et al., 2002) has been able to isolate a fucoidanase out of fungi. As our experiments and those of de la Cruz (de la Cruz et al., 2006) could not confirm his results and the results of our project partner are not distinct (Kelly et al., 2008), a fucoidan-degrading fungus still has to be found.

The results for the microorganisms were also not as promising as we had hoped. The project started under the premises of having a potent fungal strain that is able to degrade fucoidan. As the fungus did not show the wanted activity, new microorganisms had to be found and isolated as it is very difficult to purchase microorganisms that are expressing a fucoidanase. The only commercially available microorganism with a published fucoidan-degrading ability was *Saccharophagus degradans* (Gonzalez and Weiner, 2000) and even with this strain the results are contradictory. Problems arising through the cell attachment to the glass surface of the cultivation flask could be avoided by adding glass beads or other particles to the cultivation broth in order to present another surface to the bacteria besides the flask walls, leading to a concentration of the cell mass on this new particles. These could easily be removed from the cultivation broth and be further analysed. Cells attached to the flask wall are very difficult to remove. No pure enzyme solution could be produced which means that the reported fucoidan-degrading ability is based on whole-cell conversions. The growth conditions of the bacteria as well as the detection procedures have to be optimised further in order to get reliable results. In 1996 Sakai et al. (Sakai et al., 1996) patented the production of oligosaccharides manufactured by enzymatic hydrolysis.

To summarise the results of the microbial experiments it turns out that a lot of experiments still have to be conducted. There are first hints of fucoidan-degrading bacteria and fungi, but the project is still far away from the good of producing a fucoidan-degrading enzyme. New methods have to be taken into consideration, including genetic engineering in order to

overproduce such enzymes. Expanded experiments concerning the cultivation parameters should elucidate the ability of the strains to produce the desired enzyme activity. New information could be gained about the handling of the newly isolated strains as well as the commercially available ones.

Enzymes produced by the various microorganisms have to be produced in higher amount, purified and genetically analysed. In recent years more and more groups were able to find microorganisms with fucoidan-degrading abilities (Bakunina et al., 2002; Furukawa et al., 1992; Ivanova et al., 2002; Kitamura et al., 1992; Urvantseva et al., 2006). In 2008 Kim et al. (Kim et al., 2008) described a fucoidanase from *Sphingomonas paucimobilis* elucidated through 16S rDNA. The fucoidanase activity was not present in the supernatant. Which is in agreement with our experiments performed on other bacteria. Colin et al. (Colin et al., 2006) as well as Sakai et al. (Sakai et al., 2004) tried to produce genetically fucoidanases and to patent their findings. Fucoidanases are very special enzymes due to the great variety of structural motifs in fucoidan. Unfortunately, no endofucanase is commercially available yet, which Chevolot et al. (Chevolot et al., 1999) already criticised in 1999. Several research groups have found organisms that produce the desired enzyme, but not all have made their enzymes available.

Since 2008, the strain *Mesonia algae, Marini flexile fucanivorans* (Descamps et al., 2006) as well as *Pseudoalteromonas issachenkonii* (Alexeeva et al., 2002) are commercially available. *Mesonia alga* was described by Urvantseva et al. in 2006 as having been isolated from green alga *Acrosiphonia sonderi,* and together with *Maribacter sp.* and *Gramella sp*. (associates of the sea urchin *S. intermedius*), it was one of the best producers of fucoidanases. Xylose effectively induced the biosynthesis of fucoidanases in these strains (Urvantseva et al., 2006). *M. algae* is able to degrade fucoidan from *Fucus evanescens* (Urvantseva et al., 2006). These two fucoidans possess similar structural motifs (Cumashi et al., 2007). It would be interesting to see, whether *M. algae*, *Marini flexile fucanivorans* and *P. issachenkonii* are able to degrade fucoidan from *Fucus vesiculosus* isolated at the German coast.

# 6 Outlook

The production and analysis of fucoidan was successfully performed in this thesis. It would be very interesting to further elucidate the structure and composition of the produced extracts by enzymatic degradation with commercial available glucosidases.

A great potential lies in the bioactivity of FVEhigh, FVElow, Fucoidan Sigma and LDEhigh. Further analyses against other viruses are very interesting, since FVEhigh exceeds the anti-viral activity of ganciclovir which has already been successfully applied as a virus statica. The bioactivity detected against blood clotting corresponds with literature but even more detailed results should be produced. It would be very interesting to test the effect on human blood samples, but since the experience and safety regulations at our institute do not allow these kinds of experiments they would necessitate a suitable project partner. The bioactivity trials against skin cancer is still ongoing and will reveal the effect of all extracts against mouse skin cancer. As an antibody for Fucoidan Sigma could be found, it would be interesting to perform a panning against the other extracts to find antibodies for FVEhigh, FVElow and LDEhigh.

With *Pseudoalteromonas atlantica* and *Saccharophagus degradans* two microorganisms with a fucoidan-degrading potential were identified. Further cultivation experiments as well as enzymatic analysis would be of great interest. Cultivation optimisation is inevitable, especially for *Saccharophagus degradans*. Experiments with 'beads' as surface for the bacteria to grow on, thus reducing the loss of biomass due to biofilm production would be worth the trouble. Unfortunately, no fucoidanase or similar enzyme could be isolated, purified and used without the host so far. Since a genetically modified common host could potentially produce fucoidanases in greater amounts, a screening for a fucoidanase gene is needed. By now genetic data of fucoidanases are available. To elucidate the size and genetic composition of the expressed enzymes of our two interesting strains, so called 'zymograms' could be generated. In this case, the proteins of a cultivation broth (or cell disruption suspension) are separated by a native gel electrophoresis. The polysaccharides that shall be cleaved are applied as coloured version in the gel (if available). Degrading activity can be seen after development of the gel electrophoresis as the band of the degrading enzyme shows a different coloration as the rest of the gel. One could then excise the enzyme for further analysis.

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# 8 Abbreviations and Symbols

**Table 24: Abbreviations**

| Abbreviation | Meaning |
|---|---|
| AEC | 3-Amino-9-ethylcarbazole |
| AG | Arbeitsgruppe |
| APS | Ammonium peroxide sulphate |
| APU | Antigen Producing Unit |
| BSTFA | Bistrimethylsilyl-trifluoracetamid |
| CMV | Cytomegalovirus |
| C-PAGE | Carbohydrate Polyacrylamide Gel Electrophoresis |
| DEAE | Diethyl Amino Ethyl |
| DMBA | Dimethylbenz(α)anthracene |
| DMF | Dimethylformamid |
| DMSO | Dimethylesulfoxide |
| DNS | Dinitrosalicylic Acid |
| EBV-EA | Epstein-Barr-Virus early antigen |
| ELISA | Enzyme Linked Immuno Sorbent Assay |
| $E_{spec}$ | Specific energy application |
| FVEhigh | *Fucus vesiculosus* extract high molecular weight |
| FVElow | *Fucus vesiculosus* extract low molecular weight |
| GC MS | Gas Chromatography Mass Spectrometry |
| HAL4/7 | Human Antibody Library 4/7 |
| HCMV | Human Cytomegalovirus |
| HIV | Human Immunodeficiency Virus |
| HMK | High Molecular Kininogen |
| HRP | Horse Radish Peroxidase |
| ICR | Imprinting Control Region |
| IEC | Ion Exchange Chromatography |
| IgG | Immunglobulin |
| IPAT | Institut für Partikeltechnik |
| IPTG | Isopropyl-β-D-thiogalactopyranoside |
| LB | Lysogeny Broth; also known as Luria-Bertani medium |
| LDEhigh | *Laminaria digitata* extract; high molecular weight |
| M13K07 | M13 phage |
| MOI | Multiplicity Of Infection |
| MPBST | Milk Powder in PBS + 0.1% Tween 20 |
| MPY | Maltose-Peptone-Yeast |

| Abbreviation | Meaning |
|---|---|
| MRC-5 | Cell line produced for the Medical Research Council, GB; The MRC-5 cell line was developed in September 1966 from lung tissue taken from a 14-week-male-fetus aborted for psychiatric reasons from a 27-year-old physically healthy woman. The cell morphology is fibroblast-like. |
| MTBE | Methyl *tert*. –butyl ether |
| MW | Molecular Weight |
| MWCO | Molecular Weight Cut Off |
| NO | Peroxynitrite |
| P | Power |
| PAGE | Polyacrylamide Gel Electrophoresis |
| PBS | phosphate buffered saline<br>pH 7.4 (8.0 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4 \cdot 2\ H_2O$, 0.24 g $KH_2PO_4$ in 1 litre) |
| PBST | PBS + 0.1% Tween 20 |
| PFU | Plaque Forming Units |
| scFv | Single Chain Antibody Variable Fragment |
| SDS | Sodium Dodecyl Sulphate |
| SEC | Size Exclusion Chromatography |
| SEHPLC | Size exclusion high performance liquid chromatography |
| TEMED | Tetramethylethylendiamine |
| TLC | Thin Layer Chromatography |
| TM | Terra Mare; Institute for Chemistry and Biology of the Marine environment (ICBM) |
| TMB | Tetramethyl benzidine |
| TPA | 12-O-Tertradecanoylphorbol-13-acetate |
| TY-A | Tryptone Yeast Broth with 100 µg/ml ampicillin |
| TY-GA | Tryptone Yeast Extract Glucose Agar |
| TY-T | Tryptone Yeast Broth with 50 µg/ml tetracycline |
| $V_{susp}$ | Volume of the suspension |
| WHV | Wilhelmshaven |
| XL-1 Blue | *E.coli* cells from Stratagene; Δ(*mcr*A)183, Δ(*mcr*CB-*hsc*(SMR-*mrr*)173, *end*(A1), *sup*E44 *thi*-1, *rec*A1, *gyr*A96, *rel*A1, *lac*[F' *pro*AB, $lacI^q$ ZΔM15Tn10 ($Tet^r$) |
| YPD | Yeast-Peptone-Dextrose |

# 9 Appendix

In this appendix the recipes and original data of the presented experiments are stated.

## 9.1 Bradford Test

If no commercially available Bradford reagent is available, it can be mixed according to Table 25.

**Table 25: Bradford reagent**

| Chemical | Amount |
|---|---|
| Brilliant Blue | 300 mg |
| Methanol | 300 ml |
| Ortho-phosphoric acid | 600 ml |

Brilliant Blue has to be dissolved in the methanol for 30 min. After that the ortho-phosphoric acid is added. This solution is the stock solution. 150 ml of the stock solution are then diluted with 850 ml of $H_2O$ dest.. This new solution has to be filtered through a fluted filter three times. This filtered solution is the Bradford reagent which has to be further diluted for measurements.

## 9.2 DNS-Test

**Table 26: DNS-reagent**

| Chemical | Amount |
|---|---|
| Dinitrosalicylic acid | 10 g/l |
| NaOH | 16 g/l |
| kalium sodium tartrate tetra hydrate | 300 g/l |
| $H_2O$ dest. | 1000 ml |

The dinitrosalicylic acid is dissolved in NaOH in $H_2O$ dest., filtrated and kept constantly at 4 °C. The colour of the reagent has to be orange. Safety gloves have to be worn, since the agent does also react with the sugar molecules on human skin.

## 9.3 Somogyi Nelson Test

**Table 27: Alkaline copper reagent part 1**

| Chemical | Amount |
|---|---|
| Potassium sodium tatrate | 12 g |
| $Na_2CO_3$ | 24 g |
| $CuSO_4 * H_2O$ | 4 g |
| $H_2O$ dest | 300 ml |

**Table 28: Alkaline copper reagent part 2**

| Chemical | Amount |
|---|---|
| $Na_2SO_4$ | 180 g |
| $H_2O$ dest. | 500 ml |

Solution 2 is cooked for 30 min to get rid of dissolved air. Afterwards both solutions (part 1 and 2) are pooled and filled up to 1 l with $H_2O$ dest. After one week of maturation (storage) at room temperature (20 °C) the solution is ready to use.

**Table 29: Arsenic molybdate reagent part 1**

| Chemical | Amount |
|---|---|
| $(NH_4)_6Mo_7O_{24} * H_2O$ | 50 g |
| $H_2O$ dest | 950 ml |

**Table 30: Arsenic molybdate reagent part 2**

| Chemical | Amount |
|---|---|
| $H_2SO_4$ (96%) | 42 ml |
| $Na_2HSO_4 * 7H_2O$ | 6 g |

Part 1 has to be solved first, then part 2 can be added. The solution has then to be incubated at 27 °C for 3 days in a light protected surrounding.

## 9.4 SDS-PAGE

In the following the recipes for the SDS-PAGE are presented. Running is performed with 600 V, 100 W and 30 mA (per gel).

**Table 31: SDS-PAGE running gel (12%) for protein determination (total amount 4 ml)**

| Chemical | Amount |
|---|---|
| $H_2O$ dest. | 1.3 ml |
| 30% Acrylamid mix (Rotiphorese® Gel 30) | 1.6 ml |
| 1.5 M TrisHCl (pH 8.8) | 1.0 ml |
| 10% SDS | 40 µl |
| 10% APS | 40 µl |
| TEMED | 2 µl |

**Table 32: SDS-PAGE colleting gel (4%) for protein determination (total amount 1.5 ml)**

| Chemical | Amount |
|---|---|
| $H_2O$ dest. | 1.0 ml |
| 30% Acrylamid mix (Rotiphorese® Gel 30) | 0.26 ml |
| 1 M TrisHCl pH 6.8 | 0.2 ml |
| 10% SDS | 15 µl |
| 10% APS | 15 µl |
| TEMED | 2 µl |

**Table 33: Laemlli-buffer (5x); (Laemmli, 1970)**

| Chemical | Amount |
|---|---|
| glycerine | 5 ml |
| 10% SDS | 3.6 ml |
| β - Mercaptoethanol | 1.5 ml |
| Bromphenolic blue | 0.02% |

**Table 34: SDS-PAGE running buffer pH 8.3**

| Chemical | Amount (10x) | Amount (1x) |
|---|---|---|
| glycerine | 1.92 M | 192 mM |
| Tris | 250 mM = 30.3 g/L | 25 mM |
| SDS | - | 0.1% |

No pH correction is necessary if diluting from 10x buffer to 1x.

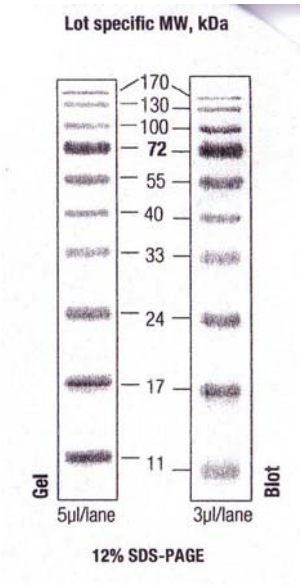


**Table 35: PageRuler™ Prestained Protein Ladder #SM0679 (Fermentas)**

## 9.5 Alcian Blue Staining Procedure

**Table 36: Alcian Blue staining solutions**

| Chemical | Amount |
|---|---|
| Alcian Blue | 0.5% |
| Acetic Acid | 2% |

Directly after gel electrophoresis the gel is put into 0.5% of Alcian Blue in a 2% acetic acid solution (or $H_2O$ dest.) and incubated for 30 min in the dark. Destaining is performed with 2% acetic acid (or $H_2O$ dest.) until the gel is clear again. This step may take several hours. Sometimes destaining over night is appropriate. The gel is washed three times with $H_2O$ dest., before silver nitrate staining is applied.

## 9.6 Silver Nitrate Staining Procedure

**Staining procedure:**

First, the collecting gel is removed from the running gel after electrophoresis, then the running gel is washed in Fixative I for 5 min. The gel can be kept in Fixative I for several days, if necessary. Afterwards, the gel is washed in Fixative II for 5 min and washed with warm water (60 °C) twice. Then a rocking step in Farmer's reducer for 30 sec is applied. The gel is then washed with warm water (60 °C) for six times. Rocking in silver nitrate staining solution takes place for 12 min. Afterwards the gel is washed with water twice. Then a rocking step in developer is applied until black spots become visible. Afterwards, the gel is washed with water again. The reaction is stopped with stopping solution. To document the gel it is photographed. The gel might be desiccated and kept. The silver nitrate staining procedure needs different solutions, which are stated in the following tables.

**Table 37: Silver nitrate staining solutions – Farmer's reducer**

| Name of the solution | Chemical | Amount |
|---|---|---|
| Farmer's Reducer | | |
| Solution A | Potassiumferrocyanid | 50 g/l |
| Solution B | Sodiumthiosulphate | 100 g/l |

Solution A and B are mixed in equal volumes shortly before usage.

**Table 38: Silver nitrate staining solutions – Fixative I**

| Name of the solution | Chemical | Amount |
|---|---|---|
| Fixative I | Methanol | 40% |
| | Acetic acid | 10% |

**Table 39: Silver nitrate staining solutions – Fixative II**

| Name of the solution | Chemical | Amount |
|---|---|---|
| Fixative II | Ethanol | 10% |
| | Acetic acid | 15% |

**Table 40: Silver nitrate staining solutions – Silver nitrate solution**

| Name of the solution | Chemical | Amount |
|---|---|---|
| Silver nitrate solution | Silver nitrate ($AgNO_3$) | 2 g/l |

**Table 41: Silver nitrate staining solutions – Developer**

| Name of the solution | Chemical | Amount |
|---|---|---|
| Developer | Sodiumcarbonate | 29 g/l |
| | Formaldehyde Solution (37%) | 1 ml/l |

**Table 42: Silver nitrate staining solutions – Stopping solution**

| Name of the solution | Chemical | Amount |
|---|---|---|
| Stopping solution | Acetic acid | 7% |

## 9.7 Elemental Analysis

In the following tables the original data from the elemental analysis at Ilse Beetz laboratory on may 22nd 2005 is shown. With these values the percentage of the different amounts is calculated.

**Table 43: Elemental analysis FVEhigh**

| | | | | | | |
|---|---|---|---|---|---|---|
| 4.090 mg total | 3.820 mg $CO_2$ | 2.040 mg $H_2O$ | 25.49% C | 5.58% H | | 0.708 mg residue (colourless, flaky) |
| 2.192 mg total | 2.903 mg J | 41.74% O | | | | |
| 15.495 mg total | 9.540 mg $BaSO_4$ | 8.45% S | | | | |

**Table 44: Elemental analysis FVElow**

| | | | | | | |
|---|---|---|---|---|---|---|
| 5.100 mg total | 6.700 mg $CO_2$ | 2.690 mg $H_2O$ | 35.85% C | 5.90% H | | 0,370 mg residue (light beige, flaky) |
| 1.761 mg total | 2.512 mg J | 44.96% O | | | | |
| 12.178 mg total | 2.320 mg $BaSO_4$ | 2.62% S | | | | |

**Table 45: Elemental analysis Fucoidan Sigma**

| | | | | | | |
|---|---|---|---|---|---|---|
| 4.512 mg total | 4.230 mg $CO_2$ | 1.720 mg $H_2O$ | 25.58% C | 4.27% H | | 1,012 mg residue (colourless-shiny+ brown) |
| 2.080 mg total | 2.428 mg J | 36.79% O | | | | |
| 12.937 mg total | 7.440 mg $BaSO_4$ | 7.90% S | | | | |

**Table 46: Elemental analysis calculated values**

| | C [%] | S [%] | H [%] | O [%] | ashes |
|---|---|---|---|---|---|
| FVEhigh | 25.49 | 8.45 | 5.58 | 41.74 | 18.74 |
| FVElow | 35.85 | 2.62 | 5.90 | 44.96 | 10.67 |
| Fucoidan Sigma | 25.58 | 7.90 | 4.27 | 36.79 | 25.46 |
| Fucose | 43.90 | 0.00 | 7.30 | 48.70 | 0.00 |
| Sulphated Fucose | 29.50 | 13.10 | 4.90 | 52.46 | 0.00 |

## 9.8 Monosaccharide Composition Determination

In Table 47 the calculated values for the monosaccharide compositions in polysaccharides is presented.

**Table 47: Calculated values for the monosaccharide composition in polysaccharides**

| monosaccharide | Fucoidan Sigma [%] | FVEhigh [%] | FVElow [%] | LDEhigh [%] | Dextran sulphate [%] |
|---|---|---|---|---|---|
| Fucose | 83 | 78 | 25 | 81 | 0 |
| Xylose | 8 | 3 | 4 | 4 | 0 |
| Mannose | 1 | 1 | 9 | 2 | 0 |
| Galactose | 7 | 8 | 4 | 12 | 0 |
| Glucose | 1 | 9 | 59 | 1 | 100 |

Figures 77 to 79 show the original data sheets for the GC/MS analyses performed on different fucoidan extractions. 0.005 g of the samples were dissolved in 0.005 l of milli-Q water.

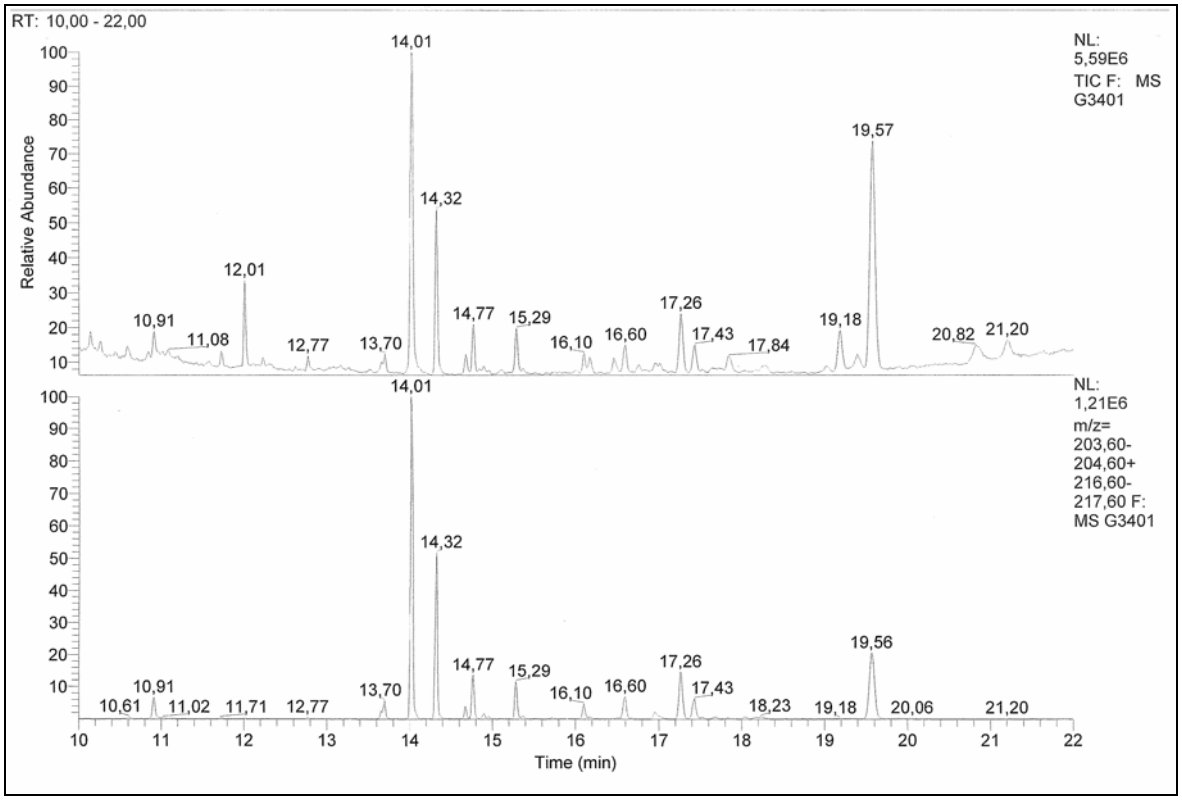


**Figure 77: GC/MS Result; FVEmixture non-neutralised**

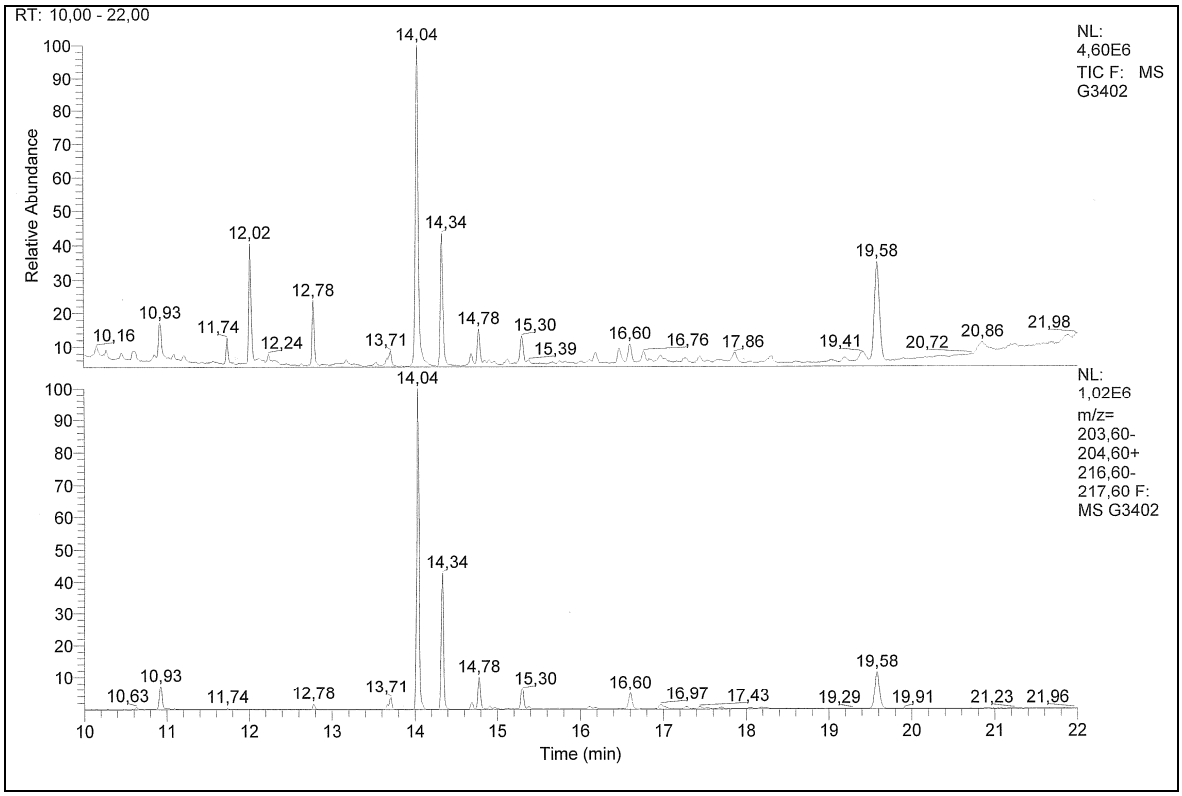


**Figure 78: GC/MS Result; FVEhigh non-neutralised**

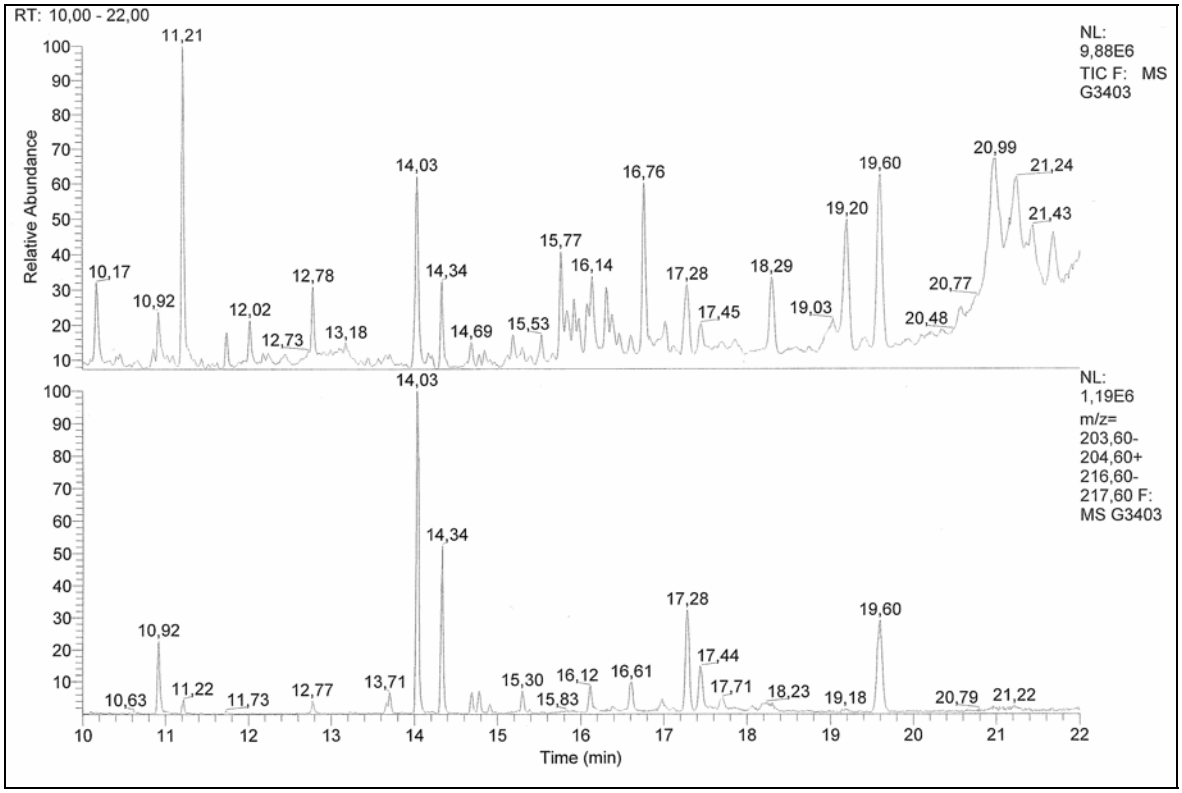


**Figure 79: GC/MS Result; FVEmixture neutralised**

## 9.9 Chemical Hydrolysis

Table 48 shows the data for the chemical hydrolysis performed on Sigma fucoidan. Parallel measurements were performed to ensure exact time intervals.

**Table 48: Fucoidan Sigma: Parallel samples for chemical hydrolysis with 0.01M HCl; Analysis by DNS Test at 540 nm.**

| Sample | Amount [g] | Time [h] | Absorption $540_{nm}$ (DNS) | Normed |
|---|---|---|---|---|
| AH-SigmaF-**1** | 0.0100 | 0.0 | 0.116 | 0.113 |
| AH-SigmaF-**2** | 0.0104 | 0.6 | 0.124 | 0.121 |
| AH-SigmaF-**3** | 0.0101 | 1.1 | 0.144 | 0.141 |
| AH-SigmaF-**4** | 0.0104 | 1.9 | 0.152 | 0.149 |
| AH-SigmaF-**5** | 0.0104 | 3.9 | 0.190 | 0.187 |
| AH-SigmaF-**6** | 0.0101 | 25.2 | 0.298 | 0.295 |
| AH-SigmaF-**7** | 0.0102 | 54.1 | 0.360 | 0.357 |
| AH-SigmaF-**8** | 0.0108 | 67.5 | 0.446 | 0.443 |
| AH-SigmaF-**9** | 0.0107 | 75.3 | 0.440 | 0.437 |
| AH-SigmaF-**10** | 0.0108 | 92.1 | 0.493 | 0.490 |
| AH-SigmaF-**11** | 0.0105 | 99.3 | 0.470 | 0.467 |
| AH-SigmaF-**12** | 0.0110 | 120.9 | 0.529 | 0.526 |
| AH-SigmaF-**13** | 0.0104 | 140.6 | 0.552 | 0.549 |
| AH-SigmaF-**14** | 0.0103 | 163.9 | 0.536 | 0.533 |
| AH-SigmaF-**15** | 0.0102 | 235.5 | 0.548 | 0.545 |
| AH-SigmaF-**16** | 0.0100 | 260.7 | 0.548 | 0.545 |
| AH-SigmaF-**17** | 0.0100 | 264.5 | 0.519 | 0.516 |
| AH-SigmaF-**18** | 0.0103 | 283.5 | 0.652 | 0.649 |
| AH-SigmaF-**19** | 0.0103 | 285.3 | 0.492 | 0.489 |
| AH-SigmaF-**20** | 0.0101 | 285.3 | 0.530 | 0.527 |
| water | | | 0.003 | 0.000 |

# 9.10 Bioactivity Data

## 9.10.1 Anti-coagulants Data

In the following tables, the anti-coagulant data collected through Hepato-Quick-test with mouse blood are presented.

**Table 49: Anti-coagulant data for several polysaccharides on mouse blood; Hepato-Quick-test, first measurement**

| substance | amount [g/ml] | dilution factor | Hepato-Quicktest* [s] | Hepato-Quicktest** [s] |
|---|---|---|---|---|
| Heparin (Fluka) | 0.0011 | - | > 999 | > 999 |
| Fucoidan Sigma | 0.0010 | - | > 999 | > 999 |
| B (Svenja) | 0.0013 | - | > 999 | > 999 |
| No substance | 0.0000 | - | 15.2 | 17.8 |
| Heparin (Fluka) | 0.0011 | 1 to 10 | > 999 | n.d. |
| Fucoidan Sigma | 0.0010 | 1 to 10 | 65.2 | n.d. |
| B (Svenja) | 0.0013 | 1 to 10 | > 999 | n.d. |
| No substance | 0.0000 | - | 20.3 | n.d. |
| Fucoidan Sigma | 0.0010 | 1 to 100 | n.d. | 16.8 |
| Heparin (Fluka) | 0.0011 | 1 to 100 | 28.7 | n.d. |
| Fucoidan Sigma | 0.0010 | 1 to 100 | 39.0 | n.d. |
| FVEhigh | 0.0013 | 1 to 100 | 18.5 | n.d. |
| FVElow | 0.0010 | 1 to 100 | 19.2 | n.d. |
| Laminarin big (Svenja) | 0.0010 | 1 to 100 | n.d. | 19.0 |
| Laminarin small (Svenja) | 0.0012 | 1 to 100 | n.d. | 20.4 |
| A (Svenja) | 0.0012 | 1 to 100 | n.d. | 20.1 |
| No substance | 0.0000 | 1 to 100 | n.d. | 20.4 |

* coagulometer No. 1; ** coagulometer No. 2; n.d. = not determined

**Table 50: Anti-coagulant data for several polysaccharides on mouse blood; Hepato-Quick-test, second measurement**

| substance | amount [g/ml] | dilution factor | Hepato-Quicktest* [s] | *** |
|---|---|---|---|---|
| No substance | 0.0000 | - | 24.0 | 1 |
| Heparin (Fluka) | 0.0011 | 1 to 50 | > 600 | 2 |
| Fucoidan Sigma | 0.0011 | 1 to 50 | 34.4 | 3 |
| FVEhigh | 0.0011 | 1 to 50 | 78.9 | 4 |
| No substance | 0.0000 | - | 24.6 | 1 |
| FVElow | 0.0011 | 1 to 50 | 18.3 | 2 |
| Laminarin (Sigma) | 0.0013 | 1 to 50 | 20.7 | 3 |
| FVEhigh hydrolysed | 0.0012 | 1 to 50 | 24.4 | 4 |
| LDEhigh | 0.0013 | 1 to 50 | > 400 | 1 |
| No substance | 0.0000 | - | 22.6 | 2 |
| LDElow | 0.0010 | 1 to 50 | 29.4 | 3 |
| A (Svenja) | 0.0012 | 1 to 50 | 68.1 | 4 |
| FVElow | 0.0010 | 1 to 50 | 24.5 | 1 |
| FVEhigh | 0.0011 | 1 to 50 | > 400 | 2 |
| No substance | 0.0000 | - | 21.3 | 3 |
| B (Svenja) | 0.0010 | 1 to 50 | 78.3 | 4 |
| Fucoidan $H_2O$ (Svenja) | 0.0012 | 1 to 50 | 63.5 | 1 |
| Fucoidan from *Laminaria* $H_2O$ (Svenja) | 0.0010 | 1 to 50 | 42.7 | 2 |
| Dextran 500,000 Da | 0.0011 | 1 to 50 | 21.5 | 3 |
| No substance | 0.0000 | - | 19.2 | 4 |

* coagulometer No. 1; *** site in the coagulometer

**Table 51: Anti-coagulant data for several polysaccharides on mouse blood; Hepato-Quick-test, third measurement**

| substance | amount [g/ml] | dilution factor | Hepato-Quicktest* [s] | *** |
|---|---|---|---|---|
| Dextran Sulphate 500,000 Da | 0.0013 | 1 to 50 | > 500 | 1 |
| Heparin (Fluka) | 0.0011 | 1 to 100 | 51.4 | 2 |
| FVEhigh | 0.0011 | 1 to 50 | 28.2 | 3 |
| Fucoidan big (Svenja) | 0.0011 | 1 to 100 | 79.0 | 4 |
| Fucoidan big (Svenja) | 0.0011 | 1 to 75 | > 600 | 1 |
| Heparin (Fluka) | 0.0011 | 1 to 75 | > 600 | 2 |
| LDEhigh | 0.0013 | 1 to 75 | 94.2 | 3 |
| No substance | 0.0000 | - | 24.6 | 4 |
| Fucoidan big (Svenja) | 0.0011 | 1 to 100 | > 500 | 1 |
| Heparin (Fluka) | 0.0011 | 1 to 100 | 45.1 | 2 |
| No substance | 0.0000 | - | 23.7 | 3 |
| LDEhigh | 0.0013 | 1 to 100 | 28.0 | 4 |
| LDElow | 0.0010 | 1 to 10 | > 500 | 1 |
| Fucoidan small (Svenja) | 0.0010 | 1 to 10 | 34.0 | 2 |
| Dextran Sulphate 500,000 Da | 0.0013 | 1 to 100 | 49.8 | 3 |

* coagulometer No. 1; *** site in the coagulometer

## 9.10.2 Anti-viral Data

All anti-viral data are presented in the results.

## 9.10.3 Anti-tumoral Data

### 9.10.3.1 In vivo two-Stage Mouse Skin Carcinogenesis Tests

**Induced with DMBA/TPA**

**Table 52: Effects of FVEhigh on *in vivo* two stage mouse skin carcinogenesis**

| | Positive control | |
|---|---|---|
| | DMBA (390 nmol) + TPA (1.7 nmol) | |
| Week | Papillomas (%) | Papillomas/ Mouse |
| 1 | 0 | 0 |
| 2 | 0 | 0 |
| 3 | 0 | 0 |
| 4 | 0 | 0 |
| 5 | 0 | 0 |
| 6 | 6.6 | 0.3 |
| 7 | 20.0 | 0.9 |
| 8 | 46.6 | 1.9 |
| 9 | 73.3 | 2.5 |
| 10 | 100 | 3.7 |
| 11 | 100 | 4.2 |
| 12 | 100 | 5.5 |
| 13 | 100 | 6.4 |
| 14 | 100 | 6.9 |
| 15 | 100 | 7.0 |
| 16 | 100 | 7.3 |
| 17 | 100 | 7.6 |
| 18 | 100 | 8.1 |
| 19 | 100 | 8.4 |
| 20 | 100 | 8.6 |

**Table 53: Effects of FVElow, FVEhigh and Fucoidan Sigma on *in vivo* two stage mouse skin carcinogenesis**

| | FVElow (85 nmol) | | FVEhigh (85 nmol) | | Fucoidan Sigma (85 nmol) | |
|---|---|---|---|---|---|---|
| Week | Papillomas (%) | Papillomas/ Mouse | Papillomas (%) | Papillomas/ Mouse | Papillomas (%) | Papillomas/ Mouse |
| 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | 13.3 | 0.7 | 13.3 | 0.6 | 6.6 | 0.4 |
| 8 | 20.0 | 1.4 | 20.0 | 1.2 | 13.3 | 0.9 |
| 9 | 26.6 | 1.8 | 26.6 | 1.7 | 13.3 | 1.5 |
| 10 | 33.3 | 2.7 | 33.3 | 2.5 | 26.6 | 2.2 |
| 11 | 40.0 | 3.6 | 33.3 | 3.4 | 33.3 | 2.9 |
| 12 | 46.6 | 4.6 | 40.0 | 4.3 | 40.0 | 3.6 |
| 13 | 53.3 | 5.4 | 53.3 | 5.2 | 46.6 | 4.2 |
| 14 | 66.6 | 6.1 | 66.6 | 5.6 | 53.3 | 5.1 |
| 15 | 73.3 | 6.5 | 73.3 | 5.9 | 60.0 | 5.5 |
| 16 | 80.0 | 6.9 | 80.0 | 6.3 | 66.6 | 5.8 |
| 17 | 86.6 | 7.2 | 86.6 | 6.8 | 73.3 | 6.3 |
| 18 | 100 | 7.5 | 93.3 | 7.1 | 80.0 | 6.5 |
| 19 | 100 | 7.8 | 100 | 7.3 | 86.6 | 6.9 |
| 20 | 100 | 8.0 | 100 | 7.7 | 93.3 | 7.1 |

## Induced with Peroxynitrite/TPA

**Table 54: Inhibitory effects of FVEhigh and curcumin on peroxynitrite–TPA-induced mouse skin carcinogenesis**

| | Positive control | | 0.0025% FVEhigh | | 0.0025 % Curcumin* | |
|---|---|---|---|---|---|---|
| | Peroxynitrite (35 µg) + TPA (1 µg) | | Two weeks oral feeding (before and after initiation) | | Two weeks oral feeding (before and after initiation) | |
| Week | Papillomas (%) | Papillomas/ Mouse | Papillomas (%) | Papillomas/ Mouse | Papillomas (%) | Papillomas/ Mouse |
| 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | 13.3 | 0.4 | 0 | 0 | 0 | 0 |
| 8 | 33.3 | 0.8 | 6.6 | 0.4 | 0 | 0 |
| 9 | 40.0 | 1.6 | 13.3 | 0.9 | 6.6 | 0.6 |
| 10 | 53.3 | 2.4 | 26.6 | 1.3 | 13.3 | 0.9 |
| 11 | 73.3 | 2.8 | 33.3 | 1.6 | 33.3 | 1.4 |
| 12 | 86.6 | 3.6 | 40.0 | 1.9 | 40.0 | 1.6 |
| 13 | 100 | 4.3 | 53.3 | 2.3 | 40.0 | 1.9 |
| 14 | 100 | 4.7 | 60.0 | 2.5 | 53.3 | 2.3 |
| 15 | 100 | 5.2 | 66.6 | 2.8 | 60.0 | 2.6 |
| 16 | 100 | 5.6 | 76.6 | 3.1 | 66.6 | 2.8 |
| 17 | 100 | 6.2 | 86.6 | 3.2 | 80.0 | 3.0 |
| 18 | 100 | 6.6 | 100 | 3.5 | 93.3 | 3.1 |
| 19 | 100 | 6.8 | 100 | 3.9 | 93.3 | 3.3 |
| 20 | 100 | 7.0 | 100 | 4.0 | 100 | 3.5 |

* Curcumin is a typical anti-inflammatory compound and is used as reference.

#### 9.10.3.2 Short term *in vitro* Bioassay for the Inhibition of Epstein Barr Virus Early Antigen (EBV-EA) Activation Induced by TPA

In Table 55 additional measurements for the inhibition potential of the EBV-EA activation of several fucoidans are presented.

**Table 55: Inhibitory effects of high-molecular weight compounds on TPA-induced EBV-EA activation**

| | Concentration (mol ratio /TPA) | | | |
|---|---|---|---|---|
| | 1000 | 500 | 100 | 10 |
| FVEhigh | 12.1 ± 0.4 (60) | 50.5 ± 1.7 | 75.5 ± 2.0 | 100.0 ± 0.8 |
| FVElow | 14.3 ± 0.5 (60) | 52.7 ± 1.9 | 78.2 ± 2.1 | 100.0 ± 0.9 |
| Fucoidan Sigma | 8.2 ± 0.3 (60 ) | 43.7 ± 1.7 | 70.2 ± 2.3 | 93. 1 ± 0.8 |

### 9.10.4 Antibody Data

The presented tables show the coating schemes and signals during panning procedures. The target or control is coated in the 96-well plate and the antibody is added.

**Table 56: General coating scheme for the 96-well ELISA plate**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | T | T | T | T | T | T | T | T | T | T | T | T |
| **B** | T | T | T | T | T | T | T | T | T | T | T | T |
| **C** | T | T | T | T | T | T | T | T | T | T | T | T |
| **D** | T | T | T | T | T | T | T | T | T | T | T | T |
| **E** | T | T | T | T | T | T | T | T | T | T | T | T |
| **F** | T | T | T | T | T | T | T | T | T | T | T | T |
| **G** | T | T | T | T | T | T | T | T | T | T | T | T |
| **H** | T | T | T | T | T | T | T | T | Lys | T | T | Lys |

T = 100 ng target/well; Lys = 100 ng lysozym/well; SH 102-10

**Table 57: ELISA-signals for Fucoidan Sigma**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | 0.21 | 0.01 | 0.14 | 0.02 | 0.01 | 0.10 | 0.03 | 0.01 | 0.04 | 0.01 | 0.01 | 0.02 |
| **B** | 0.04 | 0.01 | 0.00 | 0.02 | 0.07 | 0.01 | 0.01 | 0.04 | 2.92 | 0.04 | 0.01 | 0.02 |
| **C** | 0.01 | 0.01 | 0.00 | 0.01 | 0.01 | 0.04 | 0.02 | 0.01 | 0.04 | 0.01 | 0.01 | 0.02 |
| **D** | 0.03 | 0.01 | 0.01 | 0.01 | 0.01 | 0.02 | 0.02 | 0.01 | 0.03 | 0.02 | 0.04 | 0.03 |
| **E** | 0.02 | 0.02 | 0.00 | 0.02 | 0.01 | 0.01 | 0.01 | 0.03 | 0.01 | 0.08 | 0.02 | 0.09 |
| **F** | 0.03 | 0.04 | 0.00 | 0.00 | 0.01 | 0.2 | 0.07 | 0.03 | 0.01 | 0.11 | 0.01 | 0.01 |
| **G** | 0.00 | 0.01 | 0.00 | 0.01 | 0.01 | 0.07 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| **H** | 0.01 | 0.03 | 0.02 | 0.01 | 0.01 | 0.01 | 0.03 | 0.52 | 2.00 | 0.01 | 0.01 | 1.86 |

**Table 58: ELISA-BSA control Signals**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| **B** | 0.03 | 0.01 | 0.01 | 0.01 | 0.02 | 0.01 | 0.01 | 0.00 | 2.49 | 0.01 | 0.01 | 0.01 |
| **C** | 0.01 | 0.01 | 0.00 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| **D** | 0.03 | 0.01 | 0.01 | 0.02 | 0.02 | 0.01 | 0.01 | 0.02 | 0.01 | 0.01 | 0.02 | 0.02 |
| **E** | 0.01 | 0.01 | 0.00 | 0.01 | 0.01 | 0.01 | 0.01 | 0.02 | 0.01 | 0.01 | 0.01 | 0.02 |
| **F** | 0.0. | 0.01 | 0.00 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| **G** | 0.01 | 0.01 | 0.00 | 0.01 | 0.01 | 0.01 | 0.01 | 0.00 | 0.01 | 0.01 | 0.01 | 0.01 |
| **H** | 0.01 | 0.01 | 0.00 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 2.05 | 0.01 | 0.01 | 2.04 |

## 9.11 Cultivation Media

Several cultivation media were used during the project. Their components are given in the following tables.

**Table 59: Solid state media (Wu et al., 2002)**

| Compound | Amount |
|---|---|
| Wheat bran, straw or corn cob | 7.5 g |
| glucose | 0.5 g |
| sea water* | 6 ml |
| *Laminaria digitata* powder, FVEhigh, FVElow, Fucoidan Sigma etc. | Various amounts |

*Sea water is produced with 33 g/l sea salt in $H_2O$ dest. supplemented with 4 g/l $NaNO_3$. The inoculation of this solid state medium is done by 3ml of spore solution in 250 ml Erlenmeyer flasks.

**Table 60: Potato carrot (agar/medium) for fungus isolation**

| Compound | Amount |
|---|---|
| potato | 20 g |
| carrot | 20 g |
| salt | 33 g |
| agar | 15 g |
| $H_2O$ dest. | 1000 ml |

The potato and the carrot are cooked in boiling water until they are squashy. They are mashed through a sieve or mixed with a blender. The salt, water, and the agar are added and the mixture is autoclaved for 21 min at 121 °C. The medium can be supplemented with 0.2g/l *Laminaria digitata* powder, FVEhigh, FVElow, Fucoidan Sigma or carrageenan.

**Table 61: Biomalt (agar/medium) for fungus isolation**

| Compound | Amount |
|---|---|
| Biomalt | 50 g/l |
| Sea salt | 33 g/l |
| Agar (if needed) | 15 g/l |

pH is adjusted to 5.6 prior to autoclaving. Antibiotics are added, when the agar/medium has reached a temperature of less than 40 °C.

**Table 62: DSMZ medium 617 as isolation medium**

| Compound | Amount |
|---|---|
| Beaf extract (Lab Lemco) | 10 g/l |
| Peptone | 10 g/l |
| NaCl | 30 g/l |
| Agar | 15 g/l |

DSMZ medium 617 can be used for *Marinomonas*.

**Table 63: DSMZ medium 172 as isolation medium**

| Compound | Amount |
|---|---|
| Yeast extract (Difco) | 1 g/l |
| Tryptone (Difco) | 1 g/l |
| NaCl | 24.7 g/l |
| KCl | 0.7 g/l |
| $MgSO_4 \cdot 7H_2O$ | 6.3 g/l |
| $MgCl_2 \cdot 6\ H_2O$ | 4.6 g/l |
| $CaCl_2 \cdot 2\ H_2O$ | 1.2 g/l |
| $NaHCO_3$ | 0.2 g/l |
| Agar (Difco) | 15 g/l |

Adjust pH to 7.2. Sodium bicarbonate and calcium chloride are autoclaved separately, each in a small volume of distilled water. DSMZ medium 172 can be used for *Cytophaga*.

**Table 64: ZoBell medium as isolation medium (Baik et al., 2005; Zobell and Conn, 1940)**

| Compound | Amount |
|---|---|
| Bacto agar | 15 g/l |
| Bacto peptone | 5 g/l |
| Yeast extract | 1 g/l |
| Ferric citrate | 0.1 g/l |

**Table 65: Medium 514 (Difco 2216)**

| Compound | Amount |
|---|---|
| Bacto peptone | 5.00 g |
| Bacto yeast extract | 1.00 g |
| Fe (III) citrate | 0.10 g |
| NaCl | 19.45 g |
| $MgCl_2$ (dried) | 5.90 g |
| $NaSO_4$ | 3.24 g |
| $CaCl_2$ | 1.80 g |
| KCl | 0.55 g |
| $Na_2CO_3$ | 0.16 g |
| KBr | 0.08 g |
| $SrCl_2$ | 34.00 mg |
| $H_3BO_3$ | 22.00 mg |
| Na-silicate | 4.00 mg |
| NaF | 2.40 mg |
| $(NH_4)NO_3$ | 1.60 mg |
| $Na_2HPO_4$ | 8.00 mg |
| Distilled water | 1000 ml |

Final pH should be at 7.6 ± 0,2 at 25 °C. If using complete medium from Difco add 37,4 g to 1 litre water. Medium 514 a is medium 514 at half strength.

**Table 66: Medium 246 (Sea Water Agar)**

| Compound | Amount |
|---|---|
| Beef extract | 10.00 g |
| Peptone | 10.00 g |
| Agar | 20.00 g |
| Tap water | 250.00 ml |
| Artificial sea water | 750.00 ml |
| *Artificial sea water** | |
| NaCl | 28.13 g |
| KCl | 0.77 g |
| $CaCl_2$ x 2 $H_2O$ | 1.60 g |
| $MgCl_2$ x 6 $H_2O$ | 4.80 g |
| $NaHCO_3$ | 0.11 g |
| $MgSO_4$ x 7 $H_2O$ | 3.50 g |
| Distilled water | 1000 ml |

Dissolve beef extract and peptone by heating in tap water, adjust pH to 7.8 and boil for 10 min. Readjust pH to 7.3. Add agar and autoclave at 121 °C for 20 min. Cool to 50 °C and add warm (50 °C) sterile sea water. Liquid medium without agar should be combined when cooled to room temperature. *Natural sea water is stored in the dark for at least three weeks to "age". If natural sea water is not available use artificial sea water.

**Table 67: Fungus medium 1 (Lang et al., 2004; Lurtz and Lang, 2005)**

| Compound | Amount |
|---|---|
| NaCl | 11.5 g |
| KCl | 0.375 g |
| $CaCl_2 \cdot 2H_2O$ | 0.735 g |
| $MgCl_2 \cdot 6H_2O$ | 2.54 g |
| $MgSO_4 \cdot 7H_2O$ | 3.08 g |
| $NH_4Cl$ | 2.5 g |
| $Na_2HPO_4 \cdot 2H_2O$ | 0.445 g |
| Yeast extract | 10 g |
| Glucose | variable |
| Peptone | 10 g |
| $H_2O$ dest. | 1000 ml |

**Table 68: Synthetic sea water (Hoagland, 1933)**

| Compound | Amount |
|---|---|
| Ferric citrate | 0.1 g/l |
| NaCl | 19.45 g/l |
| $MgCl_2 \cdot 6H_2O$ | 8.8 g/l |
| $Na_2SO_4$ | 3.24 g/l |
| $CaCl_2 \cdot 2H_2O$ | 2.38 g/l |
| $Na_2HPO_4$ | 0.008 g/l |
| $SiO_2$ | 0.015 g/l |
| Trace Element Solution | 1 ml/l |
| Stock solution | 10 ml/l |

**Table 69: Trace element solution (Hoagland, 1933)**

| Compound | Amount |
|---|---|
| $H_3BO_3$ | 0.611 g/l |
| $MnCl_2 \cdot 2H^2O$ | 0.500 g/l |
| $Co(NO_3)_2 \cdot 6H_2O$ | 0.062 g/l |
| CuSO4 | 0.056 g/l |
| $ZnSO_4 \cdot 7H_2O$ | 0.056 g/l |
| $NiSO_4 \cdot 6H_2O$ | 0.056 g/l |
| $TiO_2$ | 0.056 g/l |
| $(NH_4)6Mo_7O_{24} \cdot 4H_2O$ | 0.056 g/l |
| $Al_2 (SO_4)_3 \cdot 16H_2O$ | 0.053 g/l |
| LiCl | 0.028 g/l |
| $SnCl_2$ | 0.028 g/l |
| KI | 0.028 g/l |

**Table 70: Stock solution (Hoagland, 1933)**

| Compound | Amount |
|---|---|
| KCl | 55 g/l |
| $NaHCO_3$ | 16 g/l |
| KBr | 8 g/l |
| $SrCl_2 \cdot 6H_2O$ | 3.4 g/l |
| $H_3BO_3$ | 2.2 g/l |
| NaF | 0.24 g/l |
| $NH_4NO_3$ | 0.16 g/l |

**Table 71: TY-Medium = Trypton yeast broth for antibody panning**

| Compound | Amount |
|---|---|
| Trypton | 1.6% (w/v) |
| Yeast Extract | 1% (w/v) |
| NaCl | 0.5% (w/v) |

For TY-A medium 100 µg/ml ampicillin is added, for TY-T medium 50 µg/ml tetracycline is added.

## 9.12 Bioreactor Data

Table 72 shows the original $pO_2$-data for the 8l bioreactor cultivation of Dendryphiella arenaria TM 94.

**Table 72: $pO_2$-development throughout the bioreactor (8l) cultivation of Dendryphiella arenaria TM 94 in fungi medium 1 supplemented with only 10g/l glucose.**

| Time [h] | $pO_2$ [%] | Time [h] | $pO_2$ [%] | Time [h] | $pO_2$ [%] |
|---|---|---|---|---|---|
| 0,0 | 100,0 | 41,0 | 3,0 | 60,0 | 6,0 |
| 11,0 | 100,0 | 42,0 | 3,0 | 61,0 | 5,0 |
| 12,5 | 99,0 | 43,0 | 2,0 | 62,0 | 4,0 |
| 14,0 | 98,0 | 44,0 | 2,0 | 63,0 | 11,0 |
| 18,0 | 94,0 | 45,8 | 2,0 | 64,0 | 11,0 |
| 20,0 | 90,0 | 46,0 | 46,0 | 65,0 | 14,0 |
| 22,0 | 83,0 | 46,1 | 50,0 | 66,0 | 15,0 |
| 22,0 | 91,0 | 46,3 | 46,0 | 67,0 | 19,0 |
| 22,5 | 84,0 | 46,8 | 16,0 | 68,0 | 22,0 |
| 23,0 | 82,0 | 47,0 | 40,0 | 69,0 | 26,0 |
| 24,0 | 78,0 | 48,0 | 31,0 | 69,4 | 32,0 |
| 25,0 | 71,0 | 49,0 | 26,0 | 69,5 | 11,0 |
| 26,0 | 63,0 | 50,0 | 22,0 | 70,0 | 10,0 |
| 27,0 | 52,0 | 51,0 | 18,0 | 71,0 | 13,0 |
| 28,0 | 39,0 | 51,2 | 9,0 | 72,0 | 30,0 |
| 29,0 | 23,0 | 51,4 | 22,0 | 73,0 | 35,0 |
| 29,3 | 16,0 | 52,0 | 18,0 | 74,0 | 44,0 |
| 29,8 | 74,0 | 53,0 | 15,0 | 75,0 | 49,0 |
| 30,0 | 73,0 | 54,0 | 14,0 | 76,0 | 53,0 |
| 31,0 | 66,0 | 55,0 | 12,0 | 77,0 | 56,0 |
| 32,0 | 59,0 | 56,0 | 10,0 | 84,0 | 60,0 |
| 33,0 | 51,0 | 56,0 | 22,0 | 85,0 | 67,0 |
| 34,0 | 40,0 | 56,0 | 9,0 | 86,0 | 72,0 |
| 35,0 | 28,0 | 56,5 | 11,0 | 87,0 | 74,0 |
| 36,0 | 16,0 | 56,6 | 14,0 | 91,0 | 80,0 |
| 37,0 | 10,0 | 56,8 | 12,0 | 98,0 | 83,0 |
| 38,0 | 8,0 | 57,0 | 12,0 | 105,0 | 89,0 |
| 39,0 | 6,0 | 58,0 | 8,0 | 112,0 | 92,0 |
| 40,0 | 4,0 | 59,0 | 7,0 | 113,8 | 93,0 |

## 9.13 Commercial Enzyme Tests

**Table 73: α-L-fucosidase on p-nitrophenyl-α-L-fucopyranosid**

| Time (Min and Cowman) | Absorption $405_{nm}$ | Normed Values |
|---|---|---|
| 0 | 0.225 | 0.148 |
| 5 | 1.172 | 1.095 |
| 10 | 1.492 | 1.415 |
| 15 | 1.835 | 1.758 |

# 10 Curriculum Vitae/ Lebenslauf

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